09/622857

14

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
12104-1		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB00/00763	17/05/2000	17/05/1999
International Patent Classification (IPC) or national classification and IPC		
C12N9/68		
Applicant		
CONJUCHCHEM, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	20.08.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	SCHEFFZYK, I Telephone No. +49 89 2399 8602

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB00/00763

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-67 as originally filed

Claims, No.:

1-26 with telefax of 17/05/2001

Sequence listing part of the description, pages:

1-9, filed with the letter of 17.12.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	10-26
	No:	Claims	1-9
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-26
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	26: see section VIII

2. Citations and explanations
   see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Remark: Taking into account that according to the original claims the reactive group was limited to succinimidyl and maleimido groups the search report may be incomplete with respect to newly-filed claims covering any reactive group.

SECTION V----------

WO 97/41824 (1) teaches esters of kringle 5 polypeptide derivatives. According to the definition of "reactive group" given in present application on page 4 an ester is covered by said expression. Moreover, reading the definition of "functionalities" also on page 4 of present application it is clear that ester reactive groups can form stable covalent bonds with hydroxyl groups. Correspondingly, claims 1-9 do not meet the requirements of Art. 33(2) PCT.
Moreover, the subject-matter of present claims lacks inventive activity since the principle underlying present application; i.e. the extension of the half-life of peptides within blood by adding a reactive group (anchor) to the peptides is already taught in WO 95/10302 (2) (see e.g. page 2 in combination with page 18)(a copy thereof is attached to this IPER since said document is not cited in the ISR). Correspondingly, present claims do not meet the requirements of Art. 33(3) PCT.

SECTION VI----------

WO 00/04052
WO 99/24075
WO 99/48536
WO 99/24074

SECTION VII----------

1). The sequence of the claims should be rearranged (first all product claims and subsequently the "use" claims).

2). With respect to the expression "incorporated by reference" Applicant's attention is

drawn to Guidelines C-II 4.4 and 4.17 PCT.

3). For the expression "being stable in vivo" used in newly-filed claim 25 no basis can be found in the application as filed (Art. 34(2)(b) PCT).

4). In view of Applicant's co-pending international application US00/13576 it is noted that double protection of the same subject invention basically is not possible.

SECTION VIII--------

1). The terms "modified anti-angiogenic peptide", "analog", "derivative" are vague and thus render the scope of claims containing at least one of these expressions unclear. Relating to this it is noted that the scope of a claim must be clear when seen alone, i.e. without the context of the specification.

2). Present application only evaluates the stability of kringle 5 peptides modified with maleimido groups but is silent with respect to the effect of linking kringle 5 peptides to succinimidyl group. Correspondingly, claim 17 is not technically supported by present specification (Art. 5 and 6 PCT).
Moreover, in so far as present application only exemplifies kringle 5 peptide as anti-angiogenic peptide the generalisation to any anti-angiogenic peptide is deemed unduly broad, in particular taking into account that the available prior art also only mentions kringle 5 peptides as anti-angiogenic peptides. Thus, it appears that the identification of other anti-angiogenic peptides constitutes a separate invention. Correspondingly, with respect to this term objections under Art. 5 and 6 PCT also arise.

3). Claims 26 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

WE CLAIM:

1. A modified anti-angiogenic peptide comprising a reactive group which reacts with amino groups, hydroxyl groups or thiol groups on blood components to form stable covalent bonds.

2. The as modified peptide of claim 1 wherein said peptide is a kringle 5 peptide.

3. A kringle 5 peptide according to claim 2 wherein said derivative is reactive with blood proteins.

4. A kringle 5 peptide according to claim 3, wherein the derivative is reactive with a thiol group on a blood protein.

5. A kringle 5 peptide according to claim 2 wherein the peptide is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO.:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, AND SEQ ID NO:9.

6. A kringle 5 peptide according to claim 2 wherein the peptide is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15 AND SEQ ID NO:16.

7. A composition comprising a derivative of kringle 5 peptide or analog thereof, said derivative comprising a reactive group which reacts with amino groups, hydroxyl groups or thiol groups on blood components to form stable covalent bonds for use in a method of treating angiogenesis in a human.

8. The composition of claim 7 wherein said derivative is reactive with blood proteins.

9. The composition of claim 7 wherein said derivative is reactive with a thiol group on a blood protein.

10. A derivative of a kringle 5 peptide, said derivative comprising a maleimido group which reacts with a thiol group on human serum albumin to form a covalent bond.

11. The derivative of claim 10 wherein said peptide is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9.

12. The derivative of claim 10 wherein said peptide is selected from SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15 AND SEQ ID NO:16.

13. A composition comprising a derivative of an anti-angiogenic peptide, said derivative comprising a maleimido group which reacts with a thiol group on human serum albumin to form a covalent bond for use in a method of treating angiogenesis in a human.

14. The composition of claim 13 wherein the peptide is a kringle 5 peptide.

15. The composition according to claim 14, wherein the peptide is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 AND SEQ ID NO:9.

16. A composition according to claim 14 wherein the peptide is selected from SEQ ID NO;10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15 and SEQ ID NO:16.

17. Use of a composition for the manufacturer of a medicament extending the *in vivo* half-life of a kringle 5 peptide in a patient to provide an anti-angiogenic effect, the composition comprising a derivative of a kringle 5 peptide or analog thereof, said derivative comprising a reactive group which reacts with amino groups, hydroxo groups, or thiol groups on blood components to form stable covalent bonds.

18. Use of a composition of claim 14 for the treatment of angiogenesis in a human wherein the derivative of the anti-angiogenic kringle 5 peptide is reacted with blood proteins.

19. A modified kringle 5 peptide selected from the group consisting of (MPA-AEEA)-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2 and (MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.

20. A modified kringle 5 peptide selected from the group consisting of Nac-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; MPA)-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2;NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA) - NH_2; (MPA-AEEA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; and (MPA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.

21. A modified kringle 5 peptide selected from the group consisting of NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Lys-(Nε-MPA)-NH_2; MPA-AEEA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH_2; (MPA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH_2 ; NAc-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; (MPA)-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2 ; NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Pro-Arg-Lys-Leu-Tyr-Asp-NH_2; (MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-NH_2; NAc-Pro-

Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-AEEA-MPA)-NH_2; and NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-$AEEA_n$-MPA)-NH_2.

22. A modified anti-angiogenic peptide as claimed in claim 1 wherein the reactive group comprises a succinimidyl group or a maleimido group.

23. A composition as claimed in claim 7 wherein the reactive group comprises a succinimidyl group or a maleimido group.

24. A use as claimed in claim 17 wherein the reactive group comprises a succinimidyl group or a maleimido group.

25. A conjugate comprising a modified peptide as claimed in any one of claims 1-6, 10-12 and 19-21 covalently bonded to a blood component, the conjugate being stable *in vivo*.

26. A method for extending the *in vivo* half life of an anti-angiogenic peptide comprising: coupling a reactive group to the peptide and reacting it with a functionality on a blood component to form covalent bonding, thereby forming a stable *in vivo* conjugate having an *in vivo* half-life longer than the half-life of the anti-angiogenic peptide alone.

EXPRESS MAIL LABEL NO. EL387347678US
Attorney Docket No. REDC-2200 USA

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* REDC-2200PCT

Box No. I TITLE OF INVENTION
LONG LASTING ANTI-ANGIOGENIC PEPTIDES

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

CONJUCHEM, INC.
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Canada

☐ This person is also inventor.

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Teleprinter No.

State *(that is, country)* of nationality:
CA

State *(that is, country)* of residence:
CA

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

BRIDON, Dominique P.
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This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:
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State *(that is, country)* of residence:
CA

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: MG

State *(that is, country)* of residence: CA

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: FR

State *(that is, country)* of residence: CA

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: CA

State *(that is, country)* of residence: CA

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: CA

State *(that is, country)* of residence: CA

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☒ **AP ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **TZ** United Republic of Tanzania, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian

☒ **EP European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☒ **AE** United Arab Emirates
☒ **AL** Albania
☒ **AM** Armenia
☒ **AT** Austria
☒ **AU** Australia
☒ **AZ** Azerbaijan
☒ **BA** Bosnia and Herzegovina
☒ **BB** Barbados
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☒ **BR** Brazil
☒ **BY** Belarus
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☒ **CU** Cuba
☒ **CZ** Czech Republic
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☒ **IN** India
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☒ **KG** Kyrgyzstan
☒ **KP** Democratic People's Republic of Korea
☒ **KR** Republic of Korea
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☒ **LC** Saint Lucia
☒ **LK** Sri Lanka
☒ **LR** Liberia
☒ **LS** Lesotho
☒ **LT** Lithuania
☒ **LU** Luxembourg
☒ **LV** Latvia
☒ **MA** Morocco
☒ **MD** Republic of Moldova
☒ **MG** Madagascar
☒ **MK** The former Yugoslav Republic of Macedonia
☒ **MN** Mongolia
☒ **MW** Malawi
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☒ **SK** Slovakia
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☒ **TZ** United Republic of Tanzania
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☒ **UG** Uganda
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☒ **ZW** Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

☐ .....
☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time*

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

1. If, in any of the Boxes, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:** in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication **"patent of addition,"** or **"certificate of addition,"** or if, in Box No. V, the name of the United States of America is accompanied by an indication **"continuation"** or **"continuation-in-part"**: in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI) , the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application**: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudical disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation of Box No. IV

LIMBACH, Karl A.
LIMBACH, George C.
UILKEMA, John K.
SMITH, Neil A.
DEVITT, Veronica C.
YIN, Ronald L.
SEKIMURA, Gerald T.
STALLMAN, Michael A.
GIRARD, Philip A.
POLLOCK, Michael J.
EVERETT, Stephen M.
EQUITZ, Alfred A.
SAMMUT, Charles P.
PICKERING, Mark C.
COLEMAN JAMES, Patricia
FROST, Kathleen A.
LIMBACH, Alan A.
LIMBACH, Douglas C.
OH, Seong-Kun
KING, Cameron A.
HARRIEL, Kyla L.
MAEDA, Mayumi
WARD, Michael R.
SAMPSON, Roger S.
HAMILTON, Charles L.
SMITH, Andrew V.
HOOVER, Eric N.
McCARTHY, J. Thomas
ACKERMAN, Joel G.

All attorneys are members or associates of the firm LIMBACH & LIMBACH L.L.P. Address, telephone number, and facsimile number of all are indicated in Box No. IV.

Box No. VI PRIORITY CLAI ☐ Further priority claim indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 17 May1999 (17.05.99)	60/134,406	US		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1)

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA/ EP

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country (or regional Office)

Box No. VIII CHECK LIST: LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	5
description (excluding sequence listing part)	:	67
claims	:	4
abstract	:	1
drawings	:	0
sequence listing part of description	:	9
Total number of sheets	:	86

This international application is **accompanied** by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☒ separate ~~signed~~ power of attorney (unsigned)
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: Transmittal Letter; Express Mail Certificate; Acknowledgment Card

Figure of the drawings which should accompany the abstract: ----

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

LIMBACH & LIMBACH L.L.P.

By Michael R. Ward
Michael R. Ward
Attorneys for Applicants

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA/
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PCT/IB00/00763

PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

To:

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US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

Date of mailing (day/month/year) 06 March 2001 (06.03.01)	
International application No. PCT/IB00/00763	**Applicant's or agent's file reference** 6329-51
International filing date (day/month/year) 17 May 2000 (17.05.00)	**Priority date** (day/month/year) 17 May 1999 (17.05.99)
Applicant BRIDON, Dominique, P. et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   15 December 2000 (15.12.00)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Zakaria EL KHODARY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

IB0000763

EXPRESS MAIL LABEL NO. EL387347678US
Attorney Docket No. REDC-2200 USA

PCT/IB00/00763

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

To:

RECEIVED
JUL 11 2000

WARD, Michael, R.
Limbach & Limbach L.L.P.
2001 Ferry Building
San Francisco, CA 94111-4207
ETATS-UNIS D'AMERIQUE

LIMBACH & LIMBACH LLP

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
26 June 2000 (26.06.00)	
Applicant's or agent's file reference	**International application No.**
REDC-2200PCT	PCT/IB00/00763 ✓

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

CONJUCHEM, INC. (for all designated States except US)
BRIDON, Dominique, P. et al (for US)

International filing date	:	17 May 2000 (17.05.00) ✓
Priority date(s) claimed	:	17 May 1999 (17.05.99) ✓
Date of receipt of the record copy by the International Bureau	:	09 June 2000 (09.06.00)
List of designated Offices	:	

AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
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ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [X] confirmation of precautionary designations
- [X] requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer: Eugénia Santos
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Form PCT/IB/301 (July 1998)

003373914

INFORMATION ON TIME LIMITS FOR ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated in the Notification of Receipt of Record Copy (Form PCT/IB/301) by paying national fees and furnishing translations, as prescribed by the applicable national laws.

The time limit for performing these procedural acts is **20 MONTHS** from the priority date or, for those designated States which the applicant elects in a demand for international preliminary examination or in a later election, **30 MONTHS** from the priority date, provided that the election is made before the expiration of 19 months from the priority date. Some designated (or elected) Offices have fixed time limits which expire even later than 20 or 30 months from the priority date. In other Offices an extension of time or grace period, in some cases upon payment of an additional fee, is available.

In addition to these procedural acts, the applicant may also have to comply with other special requirements applicable in certain Offices. **It is the applicant's responsibility** to ensure that the necessary steps to enter the national phase are taken in a timely fashion. Most designated Offices do not issue reminders to applicants in connection with the entry into the national phase.

For detailed information about the procedural acts to be performed to enter the national phase before each designated Office, the applicable time limits and possible extensions of time or grace periods, and any other requirements, see the relevant Chapters of Volume II of the PCT Applicant's Guide. Information about the requirements for filing a demand for international preliminary examination is set out in Chapter IX of Volume I of the PCT Applicant's Guide.

GR and ES became bound by PCT Chapter II on 7 September 1996 and 6 September 1997, respectively, and may, therefore, be elected in a demand or a later election filed on or after 7 September 1996 and 6 September 1997, respectively, regardless of the filing date of the international application. (See second paragraph above.)

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

CONFIRMATION OF PRECAUTIONARY DESIGNATIONS

This notification lists only specific designations made under Rule 4.9(a) in the request. It is important to check that these designations are correct. Errors in designations can be corrected where precautionary designations have been made under Rule 4.9(b). The applicant is hereby reminded that any precautionary designations may be confirmed according to Rule 4.9(c) before the expiration of 15 months from the priority date. If it is not confirmed, it will automatically be regarded as withdrawn by the applicant. There will be no reminder and no invitation. Confirmation of a designation consists of the filing of a notice specifying the designated State concerned (with an indication of the kind of protection or treatment desired) and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.

REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before that date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau or if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity to furnish the priority document within a time limit which is reasonable under the circumstances.

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit is the filing date of the earliest application whose priority is claimed.

PATENT COOPERATION TREA

PCT

From the INTERNATIONAL BUREAU

To:

WARD, Michael, R.
Limbach & Limbach L.L.P.
2001 Ferry Building
San Francisco, CA 94111-4207
ETATS-UNIS D'AMERIQUE

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 31 July 2000 (31.07.00)	
Applicant's or agent's file reference REDC-2200PCT	**IMPORTANT NOTIFICATION**
International application No. PCT/IB00/00763 ✓	International filing date (day/month/year) 17 May 2000 (17.05.00) ✓
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 17 May 1999 (17.05.99) ✓
Applicant CONJUCHEM, INC. et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
17 May 1999 (17.05.99)	60/134,406	US	07 July 2000 (07.07.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Khemais BRAHMI

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :		(11) International Publication Number: WO 00/70665
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(22) International Filing Date: 17 May 2000 (17.05.00)

(30) Priority Data:
60/134,406 17 May 1999 (17.05.99) US

(71) Applicant *(for all designated States except US)*: CONJUCHEM, INC. [CA/CA]; 3rd floor, Suite 3950, 225 President Kennedy Avenue West, Montreal, Quebec H2X 3Y8 (CA).

(72) Inventors; and
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(74) Agent: BERESKIN & PARR; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: LONG LASTING ANTI–ANGIOGENIC PEPTIDES

(57) Abstract

Modified anti–angiogenic peptides are disclosed. The modified peptides are capable of forming a peptidase stabilized anti–angiogenic peptide. The modified anti–angiogenic peptides, particularly modified kringle 5 peptides are capable of forming a conjugate with a blood protein. Conjugates are prepared from anti–angiogenic peptides, particularly kringle 5 peptides, by combining the peptide with a reactive functional group with a blood protein. The conjugates may be formed *in vivo* or *ex vivo*. The conjugates are administered to patients to provide an anti–angiogenic effect.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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LONG LASTING ANTI-ANGIOGENIC PEPTIDES

FIELD OF THE INVENTION

This invention relates to modified anti-angiogenic peptides. In particular, this invention relates to modified kringle 5 peptides with long duration of action for the treatment of diseases related to angiogenesis.

BACKGROUND OF THE INVENTION

Angiogenesis, the development of new blood vessels, is a highly regulated and essential process of endothelial cell growth. Although angiogenesis is a highly regulated process under normal conditions, many diseases (characterized as "angiogenic diseases") are driven by persistent unregulated angiogenesis. Unregulated, angiogenesis may either cause a particular disease directly or exascerbate an existing pathological condition. For example, ocular neovacularization has been implicated as the most common cause of blindness and dominates approximately 20 eye diseases. In certain existing conditions such as arthritis, newly formed capillary blood vessels invade the joints and destroy cartilage. In diabetes, new capillaries formed in the retina invade the vitreous, bleed, and cause blindness. Growth and metastasis of solid tumors are also angiogenesis-dependent (Folkman, J., Cancer Research, 46: 467-473 (1986), Folkman, J., Journal of the National Cancer Institute, 82: 4-6 (1989)).

Much research has been performed to identify anti-antiogenic molecules. One angiogenic molecule of particular interest is plasminogen. Of particular intererest is the kringle 5 region of plasminogen and various peptides within the kringle 5 region. Both plasminogen and the kringle 5 region of plasminogen have been shown to interfere with the angiogenic process are thus known as anti-angiogenic peptides.

While useful, kringle 5 peptides, like other peptides, suffer from rapid kidney excretion, liver metabolism, and decomposition from

endogeneous peptidases leading to very short plasma half-lives thereby reducing their usefulness as anti-angiogenic agents. As a result of their short half lives, peptides such as kringle 5 require constant infusion to reach adequate plasma levels sufficient for efficient therapy.

As a result, there is a need for long lasting anti-angiogenic peptides such as kringle 5. Such long lasting peptides would be useful in treating angiogenesis related diseases in mammals.

SUMMARY OF THE INVENTION

In order to meet these needs, the present invention is directed to modified anti-angiogenic peptides. In particular, this invention is directed to modified kringle 5 peptides. The invention relates to novel chemically reactive derivatives of anti-angiogenic peptides that can react with available functionalities on mobile blood proteins to form covalent linkages. Specifically, the invention relates to novel chemically reactive derivatives of anti-angiogenic peptides such as kringle 5 peptides that can react with available functionalities on mobile blood proteins to form covalent linkages. The chemically reactive derivatives of the anti-angiogenic peptides are capable of forming a peptidase stabilized anti-angiogenic peptide.

The invention is directed to a derivative of an anti-angiogenic peptide such as a kringle 5 peptide where the derivative comprises a reactive group which reacts with amino groups, hydroxyl groups or thiol groups on blood proteins to form stable covalent bonds. In a preferred format, the anti-angiogenic peptides include succimidyl or maleimido reactive groups.

The present invention relates to modified kringle 5 peptides and derivatives thereof and their use as anti-angiogenic agents. The kringle 5 peptides include reactive groups capable of forming a covalent bond with mobile blood proteins.

In particular, the present invention relates to the following modified kringle 5 peptides: NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Lys-NH_2;

NAc-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2; Nac-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2; NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2; NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Lys-NH_2; NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; (MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; NAc-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; MPA)-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; (MPA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Lys-(Nε-MPA)-NH_2; MPA-AEEA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH_2; (MPA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH_2; NAc-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; (MPA)-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Pro-Arg-Lys-Leu-Tyr-Asp-NH_2; (MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-NH_2; NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-AEEA-MPA)-NH_2; NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-$AEEA_n$-MPA)-NH_2; and other modified kringle 5 peptides.

The modified anti-angiogenic peptides find use in the treatment of angiogenesis in humans.

DETAILED DESCRIPTION OF THE INVENTION

To ensure a complete understanding of the invention the following definitions are provided:

Reactive Groups: Reactive groups are chemical groups capable of forming a covalent bond. Such reactive groups are coupled or bonded to an anti-angiogenic, or, more specifically, a kringle 5 peptide of interest. Reactive groups will generally be stable in an aqueous environment and will usually be carboxy, phosphoryl, or convenient acyl group, either as an ester or a mixed anhydride, or an imidate, thereby capable of forming a covalent bond with functionalities such as an amino group, a hydroxy or a thiol at the target site on mobile blood components. For the most part, the esters will involve phenolic compounds, or be thiol esters, alkyl esters, phosphate esters, or the like. Reactive groups include succimidyl and maleimido groups.

Functionalities: Functionalities are groups on blood components with which reactive groups react to form covalent bonds. Functionalities include hydroxyl groups for bonding to ester reactive groups; thiol groups for bonding to imidates and thioester groups; amino groups for bonding to carboxy, phosphoryl or acyl groups on reactive groups and carboxyl groups for bonding to amino groups.

Blood Components: Blood components may be either fixed or mobile. Fixed blood components are non-mobile blood components and include tissues, membrane receptors, interstitial proteins, fibrin proteins, collagens, platelets, endothelial cells, epithelial cells and their associated membrane and membraneous receptors, somatic body cells, skeletal and smooth muscle cells, neuronal components, osteocytes and osteoclasts and all body tissues especially those associated with the circulatory and lymphatic systems. Mobile blood components are blood

components that do not have a fixed situs for any extended period of time, generally not exceeding 5, more specifically one minute. These blood components are not membrane-associated and are present in the blood for extended periods of time and are present in a minimum concentration of at least 0.1 μg/ml. Mobile blood components include serum albumin, transferrin, ferritin and immunoglobulins such as IgM and IgG. The half-life of mobile blood components is at least about 12 hours.

Protective Groups: Protective groups are chemical moieties utilized to protect peptide derivatives from reacting with themselves. Various protective groups are disclosed herein and in U.S. 5,493,007 which is hereby incorporated by reference. Such protective groups include acetyl, fluorenylmethyloxycarbonyl (FMOC), t-butyloxycarbonyl (BOC), benzyloxycarbonyl (CBZ), and the like. The specific protected amino acids are depicted in Table 1.

TABLE 1

NATURAL AMINO ACIDS AND THEIR ABBREVIATIONS

Name	3-Letter Abbreviation	1-Letter Abbreviation	Protected Amino Acids
Alanine	Ala	A	Fmoc-Ala-OH
Arginine	Arg	R	Fmoc-Arg(Pbf)-OH
Asparagine	Asn	N	Fmoc-Asn(Trt)-OH
Aspartic acid	Asp	D	Asp(tBu)-OH
Cysteine	Cys	C	Fmoc-Cys(Trt)
Glutamic acid	Glu	E	Fmoc-Glu(tBu)-OH
Glutamine	Gln	Q	Fmoc-Gln(Trt)-OH
Glycine	Gly	G	Fmoc-Gly-OH
Histidine	His	H	Fmoc-His(Trt)-OH
Isoleucine	Ile	I	Fmoc-Ile-OH
Leucine	Leu	L	Fmoc-Leu-OH
Lysine	Lys	K	Fmoc-Lys(Mtt)-OH
Methionine	Met	M	Fmoc-Met-OH
Phenylalanine	Phe	F	Fmoc-Phe-OH
Proline	Pro	P	Fmoc-Pro-OH
Serine	Ser	S	Fmoc-Ser(tBu)-OH
Threonine	Thr	T	Fmoc-Thr(tBu)-OH
Tryptophan	Trp	W	Fmoc-Trp(Boc)-OH
Tyrosine	Tyr	Y	Boc-Tyr(tBu)-OH
Valine	Val	V	Fmoc-Val-OH

Sensitive Functional Groups – A sensitive functional group is a group of atoms that represents a potential reaction site on an anti-angiogenic peptide. If present, a sensitive functional group may be chosen as the attachment point for the linker-reactive entity modification. Sensitive functional groups include but are not limited to carboxyl, amino, thiol, and hydroxyl groups.

Modified Peptides – A modified anti-angiogenic peptide is a peptide that has been modified by attaching a reactive group, and is capable of forming a peptidase stabilized peptide through conjugation to blood components. The reactive group may be attached to the anti-angiogenic peptide either via a linking group, or optionally without using a linking group. It is also contemplated that one or more additional amino acids may be added to the anti-angiogenic peptide to facilitate

the attachment of the reactive group. Modified peptides may be administered *in vivo* such that conjugation with blood components occurs *in vivo*, or they may be first conjugated to blood components *in vitro* and the resulting peptidase stabilized peptide (as defined below) administered *in vivo*. The terms "modified anti-angiogenic peptide" and "modified peptide" may be used interchangeably in this application.

Peptidase Stabilized Anti-Angiogenic Peptides – A peptidase stabilized anti-angiogenic peptide is a modified peptide that has been conjugated to a blood component via a covalent bond formed between the reactive group of the modified peptide and the functionalities of the blood component, with or without a linking group. Peptidase stabilized peptides are more stable in the presence of peptidases *in vivo* than a non-stabilized peptide. A peptidase stabilized anti-angiogenic peptide generally has an increased half life of at least 10-50% as compared to a non-stabilized peptide of identical sequence. Peptidase stability is determined by comparing the half life of the unmodified anti-angiogenic peptide in serum or blood to the half life of a modified counterpart anti-angiogenic peptide in serum or blood. Half life is determined by sampling the serum or blood after administration of the modified and non-modified peptides and determining the activity of the peptide. In addition to determining the activity, the length of the anti-angiogenic peptide may also be measured by HPLC and Mass Spectrometry.

Linking Groups: Linking groups are chemical moieties that link or connect reactive groups to anti-angiogenic peptides. Linking groups may comprise one or more alkyl groups such as methyl, ethyl, propyl, butyl, etc. groups, alkoxy groups, alkenyl groups, alkynyl groups or amino group substituted by alkyl groups, cycloalkyl groups, polycyclic groups, aryl groups, polyaryl groups, substituted aryl groups, heterocyclic groups, and substituted heterocyclic groups. Linking groups may also comprise poly ethoxy aminoacids

such as AEA ((2-amino) ethoxy acetic acid) or a preferred linking group AEEA ([2-(2-amino)ethoxy)]ethoxy acetic acid).

DETAILED DESCRIPTION OF THE INVENTION

Taking into account these definitions, the focus of this invention is to modify anti-angiogenic peptides and particularly kringle 5 peptides to improve bio-availability, extend half-life and distribution of the peptide in vivo through conjugation of the peptide onto a protein carrier without modifying its anti-angiogenesis properties. The carrier of choice (but not limited to) for this invention would be albumin conjugated through its free thiol by a kringle 5 peptide derivatized with a maleimide moiety.

1. Kringle 5 Peptides

As used herein, the term "kringle 5" refers to the region of mammalian plasminogen having three disulfide bonds which contribute to the specific three-dimensional confirmation defined by the fifth kringle region of the mammalian plasminogen molecule. One such disulfide bond links the cysteine residues located at amino acid positions 462 and 541, a second links the cysteine residues located at amino acid positions 483 and 524 and a third links the cysteine residues located at amino acid positions 512 and 536. The amino acid sequence of a complete mammalian plasminogen molecule (the human plasminogen molecule), including its kringle 5 region, is presented in (SEQ ID NO: 1).

The term "kringle 5 peptide fragments" refers to peptides with anti-angiogenic activity of between 4 and 104 amino acids (inclusive) with a substantial sequence homology to the corresponding peptide fragment of mammalian plasminogen, an α-N-terminus at about amino acid position 443 of intact mammalian plasminogen and an α-C-terminus at about position 546 of SEQ ID NO:1; an α-N-terminus at about amino acid position 513 of intact mammalian plasminogen and an α-C-terminus at about position 523 of SEQ ID NO:1; an α-N-terminus at

about amino acid position 525 of intact mammalian plasminogen and an α-C-terminus at about position 535 of SEQ ID NO:1; an α-N-terminus at about amino acid position 529 of intact mammalian plasminogen and an α-C-terminus at about position 535 of SEQ ID NO:1; an α-N-terminus at about amino acid position 529 of intact mammalian plasminogen and an α-C-terminus at about position 534 of SEQ ID NO:1 and an α-N-terminus at about amino acid position 150 of intact mammalian plasminogen and an α-C-terminus at about position 156 of SEQ ID NO:1.

In a preferred format, the kringle 5 peptide of the invention has one or more of the following sequences:

Pro-Arg-Lys-Leu-Tyr-Asp-Lys-NH_2 (SEQ ID NO:2);

Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2 (SEQ ID NO:3);

Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2 (SEQ ID NO:4);

Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Lys-NH_2 (SEQ ID NO:5);

Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2 (SEQ ID NO:6);

Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2 (SEQ ID NO:7);

Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2 (SEQ ID NO:8);

Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2 (SEQ ID NO:9);

Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2 (SEQ ID NO:10);

Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2 (SEQ ID NO:11);

Pro-Arg-Lys-Leu-Tyr-Asp-Lys-NH_2 (SEQ ID NO:12);

Pro-Arg-Lys-Leu-Tyr-Asp-NH_2 (SEQ ID NO:13);

Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2 (SEQ ID NO:14);

Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2 (SEQ ID NO:15) and

Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp (SEQ ID NO:16).

Thus, it is to be understood that the present invention is contemplated to encompass any derivatives or modifications of kringle 5 peptide fragments which have anti-angiogenic activity and includes the entire class of kringle 5 peptide fragments described herein and derivatives and modifications of those kringle 5 peptide fragments.

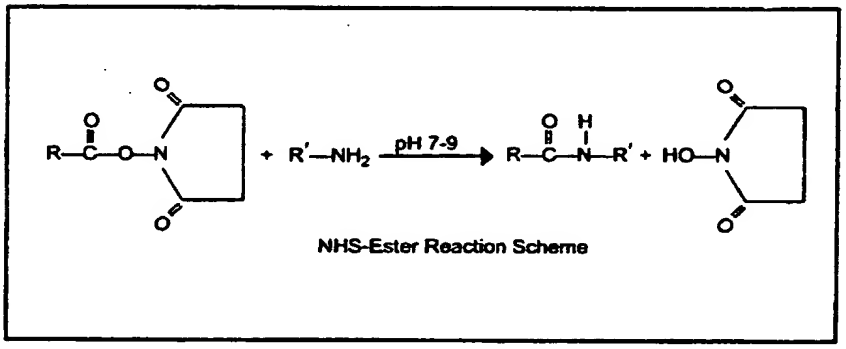
2. Modified Kringle 5 Peptides

This invention relates to modified anti-angiogenic peptides and, in particular, modified kringle 5 peptides. The modified kringle 5 peptides of the invention can react with available reactive functionalities on blood components via covalent linkages. The invention also relates to such modifications, such combinations with blood components and methods for their use. These methods include extending the effective therapeutic *in vivo* half life of the modified kringle 5 peptides.

To form covalent bonds with the functional group on a protein, one may use as a chemically reactive group (reactive entity) a wide variety of active carboxyl groups, particularly esters, where the hydroxyl moiety is physiologically acceptable at the levels required to modify the kringle 5 peptide. While a number of different hydroxyl groups may be employed in these linking agents, the most convenient would be N-

hydroxysuccinimide (NHS), N-hydroxy-sulfosuccinimide (sulfo-NHS), maleimide-benzoyl-succinimide (MBS), gamma-maleimido-butyryloxy succinimide ester (GMBS) and maleimidopropionic acid (MPA).

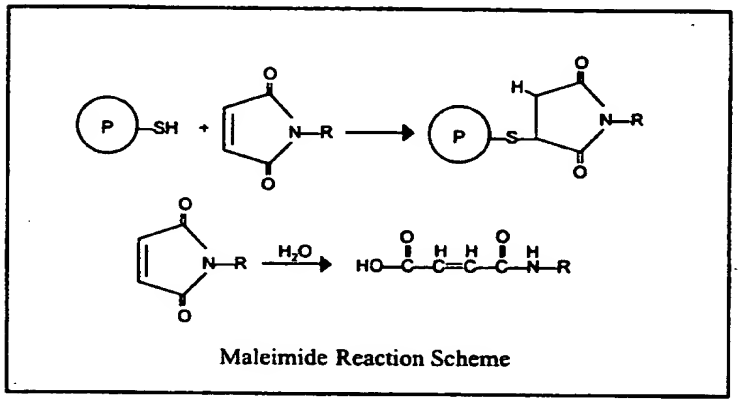
Primary amines are the principal targets for NHS esters as diagramed in the schematic below. Accessible α-amine groups present on the N-termini of proteins react with NHS esters. However, α-amino groups on a protein may not be desirable or available for the NHS coupling. While five amino acids have nitrogen in their side chains, only the ε-amine of lysine reacts significantly with NHS esters. An amide bond is formed when the NHS ester conjugation reaction reacts with primary amines releasing N-hydroxysuccinimide as demonstrated in the schematic below. These succinimide containing reactive groups are herein referred to as succinimidyl groups.



NHS-Ester Reaction Scheme

In the preferred embodiments of this invention, the functional group on the protein will be a thiol group and the chemically reactive group will be a maleimido-containing group such as gamma-maleimide butyrlamide (GMBA) or MPA. Such maleimide containing reactive groups are herein referred to as "maleimido groups." The maleimido group is most selective for sulfhydryl groups on peptides when the pH of the reaction mixture is kept between 6.5 and 7.4. At pH 7.0, the rate of reaction of maleimido groups with sulfhydryls is 1000-fold faster than

with amines. A stable thioether linkage between the maleimido group and the sulfhydryl is formed which cannot be cleaved under physiological conditions as demonstrated in the following schematic.



Maleimide Reaction Scheme

The kringle 5 peptides and peptide derivatives of the invention may be modified for specific labeling and non-specific labeling of blood components.

A. **Specific Labeling**

Preferably, the modified angiogenic peptides of this invention are designed to specifically react with thiol groups on mobile blood proteins. Such reaction is preferably established by covalent bonding of a anti-angiogenic peptide modified with a maleimide link (e.g. prepared from GMBS, MPA or other maleimides) to a thiol group on a mobile blood protein such as serum albumin or IgG.

Under certain circumstances, specific labeling with maleimides (maleimido groups) offers several advantages over non-specific labeling of mobile proteins with groups such as NHS and sulfo-NHS. Thiol groups are less abundant *in vivo* than amino groups. Therefore, the maleimide derivatives of this invention will covalently bond to fewer proteins. For example, in albumin (the most abundant blood protein) there is only a single thiol group. Thus, peptide-maleimide-albumin

conjugates will tend to comprise approximately a 1:1 molar ratio of peptide to albumin. In addition to albumin, IgG molecules (class II) also have free thiols. Since IgG molecules and serum albumin make up the majority of the soluble protein in blood they also make up the majority of the free thiol groups in blood that are available to covalently bond to maleimide-modified peptides.

Further, even among free thiol-containing blood proteins, specific labeling with maleimides leads to the preferential formation of peptide-maleimide-albumin conjugates, due to the unique characteristics of albumin itself. The single free thiol group of albumin, highly conserved among species, is located at amino acid residue 34 (Cys^{34}). It has been demonstrated recently that the Cys^{34} of albumin has increased reactivity relative to free thiols on other free thiol-containing proteins. This is due in part to the very low pK value of 5.5 for the Cys^{34} of albumin. This is much lower than typical pK values for cysteines residues in general, which are typically about 8. Due to this low pK, under normal physiological conditions Cys^{34} of albumin is predominantly in the ionized form, which dramatically increases its reactivity. In addition to the low pK value of Cys^{34}, another factor which enhances the reactivity of Cys^{34} is its location, which is in a crevice close to the surface of one loop of region V of albumin. This location makes Cys^{34} very available to ligands of all kinds, and is an important factor in Cys^{34}'s biological role as free radical trap and free thiol scavenger. These properties make Cys^{34} highly reactive with maleimide peptides, and the reaction rate acceleration can be as much as 1000-fold relative to rates of reaction of maleimide peptides with other free-thiol containing proteins.

Another advantage of peptide-maleimide-albumin conjugates is the reproducibility associated with the 1:1 loading of peptide to albumin specifically at Cys^{34}. Other techniques, such as glutaraldehyde, DCC, EDC and other chemical activations of, for example, free amines lack this selectivity. For example, albumin contains 52 lysine residues, 25-30 of which are located on the surface of albumin and accessible for

conjugation. Activating these lysine residues, or alternatively modifying peptides to couple through these lysine residues, results in a heterogenous population of conjugates. Even if 1:1 molar ratios of peptide to albumin are employed, the yield will consist of multiple conjugation products, some containing 0, 1, 2 or more peptides per albumin, and each having peptides randomly coupled at any one of the 25-30 available lysine sites. Given the numerous combinations possible, characterization of the exact composition and nature of each batch becomes difficult, and batch-to-batch reproducibility is all but impossible, making such conjugates less desirable as anti-angiogenic peptides. Additionally, while it would seem that conjugation through lysine residues of albumin would at least have the advantage of delivering more anti-angiogenic agent per albumin molecule, studies have shown that a 1:1 ratio of anti-angiogenic agent to albumin is preferred. In an article by Stehle, et al., "The Loading Rate Determines Tumor Targeting Properties of Methotrexate-Albumin Conjugates in Rats," Anti-Cancer Drugs, Vol. 8, pp. 677-685 (1997), incorporated herein in its entirety, the authors report that a 1:1 ratio of the anti-cancer methotrexate to albumin conjugated via glutaraldehyde gave the most promising results. These conjugates were taken up by tumor cells, whereas conjugates bearing 5:1 to 20:1 methotrexate molecules had altered HPLC profiles and were quickly taken up by the liver *in vivo*. It is postulated that at these higher ratios, conformational changes to albumin diminish its effectiveness as a therapeutic carrier.

Through controlled administration of maleimide-peptides *in vivo*, one can control the specific labeling of albumin and IgG *in vivo*. In typical administrations, 80-90% of the administered maleimide-peptides will label albumin and less than 5% will label IgG. Trace labeling of free thiols such as glutathione will also occur. Such specific labeling is preferred for *in vivo* use as it permits an accurate calculation of the estimated half-life of the administered agent.

In addition to providing controlled specific *in vivo* labeling, maleimide-peptides can provide specific labeling of serum albumin and IgG *ex vivo*. Such *ex vivo* labeling involves the addition of maleimide-peptides to blood, serum or saline solution containing serum albumin and/or IgG. Once modified *ex vivo* with maleimide-peptides, the blood, serum or saline solution can be readministered to the blood for *in vivo* treatment.

In contrast to NHS-peptides, maleimide-peptides are generally quite stable in the presence of aqueous solutions and in the presence of free amines. Since maleimide-peptides will only react with free thiols, protective groups are generally not necessary to prevent the maleimide-peptides from reacting with itself. In addition, the increased stability of the peptide permits the use of further purification steps such as HPLC to prepare highly purified products suitable for *in vivo* use. Lastly, the increased chemical stability provides a product with a longer shelf life.

B. **Non-Specific Labeling.**

The kringle 5 peptides of the invention may also be modified for non-specific labeling of blood components. Bonds to amino groups will also be employed, particularly with the formation of amide bonds for non-specific labeling. To form such bonds, one may use as a chemically reactive group coupled to the kringle 5 peptide a wide variety of active carboxyl groups, particularly esters, where the hydroxyl moiety is physiologically acceptable at the levels required. While a number of different hydroxyl groups may be employed in these linking agents, the most convenient would be N-hydroxysuccinimide (NHS) and N-hydroxy-sulfosuccinimide (sulfo-NHS), which form succinimidyl groups.

Other linking agents which may be utilized are described in U.S. Patent 5,612,034, which is hereby incorporated herein.

The various sites with which the chemically reactive group of the subject non-specific kringle 5 peptide derivatives may react in vivo include cells, particularly red blood cells (erythrocytes) and platelets,

and proteins, such as immunoglobulins, including IgG and IgM, serum albumin, ferritin, steroid binding proteins, transferrin, thyroxin binding protein, α-2-macroglobulin, and the like. Those receptors with which the derivatized kringle 5 peptides react, which are not long-lived, will generally be eliminated from the human host within about three days. The proteins indicated above (including the proteins of the cells) will remain at least three days, and may remain five days or more (usually not exceeding 60 days, more usually not exceeding 30 days) particularly as to the half life, based on the concentration in the blood.

For the most part, reaction will be with mobile components in the blood, particularly blood proteins and cells, more particularly blood proteins and erythrocytes. By "mobile" is intended that the component does not have a fixed situs for any extended period of time, generally not exceeding 5, more usually one minute, although some of the blood component may be relatively stationary for extended periods of time. Initially, there will be a relatively heterogeneous population of functionalized proteins and cells. However, for the most part, the population within a few days will vary substantially from the initial population, depending upon the half-life of the functionalized proteins in the blood stream. Therefore, usually within about three days or more, IgG will become the predominant functionalized protein in the blood stream.

Usually, by day 5 post-administration, IgG, serum albumin and erythrocytes will be at least about 60 mole %, usually at least about 75 mole %, of the conjugated components in blood, with IgG, IgM (to a substantially lesser extent) and serum albumin being at least about 50 mole %, usually at least about 75 mole %, more usually at least about 80 mole %, of the non-cellular conjugated components.

Preferably, the kringle 5 peptide derivative is conjugated to albumin.

The desired conjugates of non-specific kringle 5 peptides to blood components may be prepared *in vivo* by administration of the kringle 5

peptide derivatives to the patient, which may be a human or other mammal. The administration may be done in the form of a bolus or introduced slowly over time by infusion using metered flow or the like.

If desired, the subject conjugates may also be prepared *ex vivo* by combining blood with derivatized kringle 5 peptides of the present invention, allowing covalent bonding of the derivatized kringle 5 peptides to reactive functionalities on blood components and then returning or administering the conjugated blood to the host. Moreover, the above may also be accomplished by first purifying an individual blood component or limited number of components, such as red blood cells, immunoglobulins, serum albumin, or the like, and combining the component or components *ex vivo* with the chemically reactive kringle 5 peptide derivatives. The functionalized blood or blood component may then be returned to the host to provide *in vivo* the subject therapeutically effective conjugates. The blood also may be treated to prevent coagulation during handling *ex vivo*.

3. Synthesis of Modified Kringle 5 Peptides

A. Kringle 5 Peptide Synthesis

Kringle 5 peptide fragments may be synthesized by standard methods of solid phase peptide chemistry known to those of ordinary skill in the art. For example, kringle 5 peptide fragments may be synthesized by solid phase chemistry techniques following the procedures described by Steward and Young (Steward, J. M. and Young, J. D., Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Company, Rockford, Ill., (1984) using an Applied Biosystem synthesizer. Similarly, multiple fragments may be synthesized then linked together to form larger fragments. These synthetic peptide fragments can also be made with amino acid substitutions at specific locations.

For solid phase peptide synthesis, a summary of the many techniques may be found in J. M. Stewart and J. D. Young, Solid Phase Peptide Synthesis, W. H. Freeman Co. (San Francisco), 1963 and J.

Meienhofer, Hormonal Proteins and Peptides, vol. 2, p. 46, Academic Press (New York), 1973. For classical solution synthesis see G. Schroder and K. Lupke, The Peptides, Vol. 1, Acacemic Press (New York). In general, these methods comprise the sequential addition of one or more amino acids or suitably protected amino acids to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid is protected by a suitable protecting group. The protected or derivatized amino acid is then either attached to an inert solid support or utilized in solution by adding the next amino acid in the sequence having the complimentary (amino or carboxyl) group suitably protected and under conditions suitable for forming the amide linkage. The protecting group is then removed from this newly added amino acid residue and the next amino acid (suitably protected) is added, and so forth.

After all the desired amino acids have been linked in the proper sequence, any remaining protecting groups (and any solid support) are removed sequentially or concurrently to afford the final polypeptide. By simple modification of this general procedure, it is possible to add more than one amino acid at a time to a growing chain, for example, by coupling (under conditions which do not racemize chiral centers) a protected tripeptide with a properly protected dipeptide to form, after deprotection, a pentapeptide.

A particularly preferred method of preparing compounds of the present invention involves solid phase peptide synthesis wherein the amino acid α-N-terminal is protected by an acid or base sensitive group. Such protecting groups should have the properties of being stable to the conditions of peptide linkage formation while being readily removable without destruction of the growing peptide chain or racemization of any of the chiral centers contained therein. Suitable protecting groups are 9-fluorenylmethyloxycarbonyl (Fmoc), t-butyloxycarbonyl (Boc), benzyloxycarbonyl (Cbz), biphenylisopropyloxycarbonyl , t-amyloxycarbonyl, isobornyloxycarbonyl, α, α-dimethyl-3,5-dimethoxybenzyloxycarbonyl, o-nitrophenylsulfenyl, 2-cyano-t-

butyloxycarbonyl, and the like. The 9-fluorenyl-methyloxycarbonyl (Fmoc) protecting group is particularly preferred for the synthesis of kringle 5 peptide fragments. Other preferred side chain protecting groups are, for side chain amino groups like lysine and arginine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (pmc), nitro, p-toluenesulfonyl, 4-methoxybenzene-sulfonyl, Cbz, Boc, and adamantyloxycarbonyl; for tyrosine, benzyl, o-bromobenzyloxycarbonyl, 2,6-dichlorobenzyl, isopropyl, t-butyl (t-Bu), cyclohexyl, cyclopenyl and acetyl (Ac); for serine, t-butyl, benzyl and tetrahydropyranyl; for histidine, trityl, benzyl, Cbz, p-toluenesulfonyl and 2,4-dinitrophenyl; for tryptophan, formyl; for asparticacid and glutamic acid, benzyl and t-butyl and for cysteine, triphenylmethyl (trityl).

In the solid phase peptide synthesis method, the α-C-terminal amino acid is attached to a suitable solid support or resin. Suitable solid supports useful for the above synthesis are those materials which are inert to the reagents and reaction conditions of the stepwise condensation-deprotection reactions, as well as being insoluble in the media used. The preferred solid support for synthesis of $_{\alpha}$-C-terminal carboxy peptides is 4-hydroxymethylphenoxymethyl-copoly(styrene-1% divinylbenzene). The preferred solid support for $_{\alpha}$ -C-terminal amide peptides is the 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamidoethyl resin available from Applied Biosystems (Foster City, Calif.). The α-C-terminal amino acid is coupled to the resin by means of N,N'-dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide (DIC) or O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU), with or without 4-dimethylaminopyridine (DMAP), 1-hydroxybenzotriazole (HOBT), benzotriazol-1-yloxy-tris(dimethylamino)phosphonium-hexafluorophosphate (BOP) or bis(2-oxo-3-oxazolidinyl)phosphine chloride (BOPCl), mediated coupling for from about 1 to about 24 hours at a temperature of between 10° and 50° C. in a solvent such as dichloromethane or DMF.

When the solid support is 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-acetamidoethyl resin, the Fmoc group is cleaved with a secondary amine, preferably piperidine, prior to coupling with the α-C-terminal amino acid as described above. The preferred method for coupling to the deprotected 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-acetamidoethyl resin is O-benzotriazol-1-yl-N,N,N',N'-tetramethyluroniumhexafluoro-phosphate (HBTU, 1 equiv.) and 1-hydroxybenzotriazole (HOBT, 1 equiv.) in DMF. The coupling of successive protected amino acids can be carried out in an automatic polypeptide synthesizer as is well known in the art. In a preferred embodiment, the α-N-terminal amino acids of the growing peptide chain are protected with Fmoc. The removal of the Fmoc protecting group from the α-N-terminal side of the growing peptide is accomplished by treatment with a secondary amine, preferably piperidine. Each protected amino acid is then introduced in about 3-fold molar excess, and the coupling is preferably carried out in DMF. The coupling agent is normally O-benzotriazol-1-yl-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU, 1 equiv.) and 1-hydroxybenzotriazole (HOBT, 1 equiv.).

At the end of the solid phase synthesis, the polypeptide is removed from the resin and deprotected, either in successively or in a single operation. Removal of the polypeptide and deprotection can be accomplished in a single operation by treating the resin-bound polypeptide with a cleavage reagent comprising thianisole, water, ethanedithiol and trifluoroacetic acid. In cases wherein the α-C-terminal of the polypeptide is an alkylamide, the resin is cleaved by aminolysis with an alkylamine. Alternatively, the peptide may be removed by transesterification, e.g. with methanol, followed by aminolysis or by direct transamidation. The protected peptide may be purified at this point or taken to the next step directly. The removal of the side chain protecting groups is accomplished using the cleavage cocktail described above. The fully deprotected peptide is purified by a sequence of

chromatographic steps employing any or all of the following types: ion exchange on a weakly basic resin (acetate form); hydrophobic adsorption chromatography on underivitized polystyrene-divinylbenzene (for example, Amberlite XAD); silica gel adsorption chromatography; ion exchange chromatography on carboxymethylcellulose; partition chromatography, e.g. on Sephadex G-25, LH-20 or countercurrent distribution; high performance liquid chromatography (HPLC), especially reverse-phase HPLC on octyl- or octadecylsilyl-silica bonded phase column packing.

Molecular weights of these kringle 5 peptides are determined using Fast Atom Bombardment (FAB) Mass Spectroscopy.

The kringle 5 peptides of the invention may be synthesized with N- and C-terminal protecting groups.

1. **N-Terminal Protective Groups.**

The term "N-protecting group" refers to those groups intended to protect the α-N-terminal of an amino acid or peptide or to otherwise protect the amino group of an amino acid or peptide against undesirable reactions during synthetic procedures. Commonly used N-protecting groups are disclosed in Greene, "Protective Groups In Organic Synthesis," (John Wiley & Sons, New York (1981)), which is hereby incorporated by reference. Additionally, protecting groups can be used as pro-drugs which are readily cleaved in vivo, for example, by enzymatic hydrolysis, to release the biologically active parent. α-N-protecting groups comprise loweralkanoyl groups such as formyl, acetyl ("Ac"), propionyl, pivaloyl, t-butylacetyl and the like; other acyl groups include 2-chloroacetyl, 2-bromoacetyl, trifluoroacetyl, trichloroacetyl, phthalyl, o-nitrophenoxyacetyl, -chlorobutyryl, benzoyl, 4-chlorobenzoyl, 4-bromobenzoyl, 4-nitrobenzoyl and the like; sulfonyl groups such as benzenesulfonyl, p-toluenesulfonyl and the like; carbamate forming groups such as benzyloxycarbonyl, p-chlorobenzyloxycarbonyl, p-methoxybenzyloxycarbonyl, p-nitrobenzyloxycarbonyl, 2-

nitrobenzyloxycarbonyl, p-bromobenzyloxycarbonyl, 3,4-dimethoxybenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 2,4-dimethoxybenzyloxycarbonyl, 4-ethoxybenzyloxycarbonyl, 2-nitro-4,5-dimethoxybenzyloxycarbonyl, 3,4,5-trimethoxybenzyloxycarbonyl, 1-(p-biphenylyl)-1-methylethoxycarbonyl, α, α-dimethyl-3,5-dimethoxybenzyloxycarbonyl, benzhydryloxycarbonyl, t-butyloxycarbonyl, diisopropylmethoxycarbonyl, isopropyloxycarbonyl, ethoxycarbonyl, methoxycarbonyl, allyloxycarbonyl, 2,2,2,-trichloroethoxycarbonyl, phenoxycarbonyl, 4-nitrophenoxycarbonyl, fluorenyl-9-methoxycarbonyl, cyclopentyloxycarbonyl, adamantyloxycarbonyl, cyclohexyloxycarbonyl, phenylthiocarbonyl and the like; arylalkyl groups such as benzyl, triphenylmethyl, benzyloxymethyl, 9-fluorenylmethyloxycarbonyl (Fmoc) and the like and silyl groups such as trimethylsilyl and the like.

Preferred N-protecting groups are formyl, acetyl, benzoyl, pivaloyl, t-butylacetyl, phenylsulfonyl, benzyl, t-butyloxycarbonyl (Boc) and benzyloxycarbonyl (Cbz). For example, lysine may be protected at the α-N-terminal by an acid labile group (e.g. Boc) and protected at the -N-terminal by a base labile group (e.g. Fmoc) then deprotected selectively during synthesis.

2. **Carboxyl Protective Groups.**

The term "carboxyl protecting group" refers to a carboxylic acid protecting ester or amide group employed to block or protect the carboxylic acid functionality while the reactions involving other functional sites of the compound are performed. Carboxy protecting groups are disclosed in Greene, "Protective Groups in Organic Synthesis" pp. 152-186 (1981), which is hereby incorporated by reference. Additionally, a carboxy protecting group can be used as a pro-drug whereby the carboxy protecting group can be readily cleaved in vivo, for example by enzymatic hydrolysis, to release the biologically active parent. Such carboxy protecting groups are well known to those skilled in the art,

having been extensively used in the protection of carboxyl groups in the penicillin and cephalosporin fields as described in U.S. Pat. Nos. 3,840,556 and 3,719,667, the disclosures of which are hereby incorporated herein by reference. Representative carboxy protecting groups are C_1 -C_8 loweralkyl (e.g., methyl, ethyl or t-butyl and the like); arylalkyl such as phenethyl or benzyl and substituted derivatives thereof such as alkoxybenzyl or nitrobenzyl groups and the like; arylalkenyl such as phenylethenyl and the like; aryl and substituted derivatives thereofsuch as 5-indanyl and the like; dialkylaminoalkyl such as dimethylaminoethyl and the like); alkanoyloxyalkyl groups such as acetoxymethyl, butyryloxymethyl, valeryloxymethyl, isobutyryloxymethyl, isovaleryloxymethyl, 1-(propionyloxy)-1-ethyl, 1-(pivaloyloxyl)-1-ethyl, 1-methyl-1-(propionyloxy)-1-ethyl, pivaloyloxymethyl, propionyloxymethyl and the like; cycloalkanoyloxyalkyl groups such as cyclopropylcarbonyloxymethyl, cyclobutylcarbonyloxymethyl, cyclopentylcarbonyloxymethyl, cyclohexylcarbonyloxymethyl and the like; aroyloxyalkyl such as benzoyloxymethyl, benzoyloxyethyl and the like; arylalkylcarbonyloxyalkyl such as benzylcarbonyloxymethyl, 2-benzylcarbonyloxyethyl and the like; alkoxycarbonylalkyl or cycloalkyloxycarbonylalkyl such as methoxycarbonylmethyl, cyclohexyloxycarbonylmethyl, 1-methoxycarbonyl-1-ethyl and the like; alkoxycarbonyloxyalkyl or cycloalkyloxycarbonyloxyalkyl such as methoxycarbonyloxymethyl, t-butyloxycarbonyloxymethyl, 1-ethoxycarbonyloxy-1-ethyl, 1-cyclohexyloxycarbonyloxy-1-ethyl and the like; aryloxycarbonyloxyalkyl such as 2-(phenoxycarbonyloxy)ethyl, 2-(5-indanyloxycarbonyloxy)ethyl and the like; alkoxyalkylcarbonyloxyalkyl such as 2-(1-methoxy-2-methylpropan-2-oyloxy)ethyl and like; arylalkyloxycarbonyloxyalkyl such as 2-(benzyloxycarbonyloxy)ethyl and the like; arylalkenyloxycarbonyloxyalkyl such as 2-(3-phenylpropen-2-yloxycarbonyloxy)ethyl and the like; alkoxycarbonylaminoalkyl such as t-butyloxycarbonylaminomethyl and the like; alkylaminocarbonylaminoalkyl such as

methylaminocarbonylaminomethyl and the like; alkanoylaminoalkyl such as acetylaminomethyl and the like; heterocycliccarbonyloxyalkyl such as 4-methylpiperazinylcarbonyloxymethyl and the like; dialkylaminocarbonylalkyl such as dimethylaminocarbonylmethyl, diethylaminocarbonylmethyl and the like; (5-(loweralkyl)-2-oxo-1,3-dioxolen-4-yl)alkyl such as (5-t-butyl-2-oxo-1,3-dioxolen-4-yl)methyl and the like; and (5-phenyl-2-oxo-1,3-dioxolen-4-yl)alkyl such as (5-phenyl-2-oxo-1,3-dioxolen-4-yl)methyl and the like.

Representative amide carboxy protecting groups are aminocarbonyl and loweralkylaminocarbonyl groups.

Preferred carboxy-protected compounds of the invention are compounds wherein the protected carboxy group is a loweralkyl, cycloalkyl or arylalkyl ester, for example, methyl ester, ethyl ester, propyl ester, isopropyl ester, butyl ester, sec-butyl ester, isobutyl ester, amyl ester, isoamyl ester, octyl ester, cyclohexyl ester, phenylethyl ester and the like or an alkanoyloxyalkyl, cycloalkanoyloxyalkyl, aroyloxyalkyl or an arylalkylcarbonyloxyalkyl ester. Preferred amide carboxy protecting groups are loweralkylaminocarbonyl groups. For example, aspartic acid may be protected at the α-C-terminal by an acid labile group (e.g. t-butyl) and protected at the β-C-terminal by a hydrogenation labile group (e.g. benzyl) then deprotected selectively during synthesis.

B. Modification of Kringle 5 Peptides

The manner of producing the modified kringle 5 peptides of the present invention will vary widely, depending upon the nature of the various elements comprising the molecule. The synthetic procedures will be selected so as to be simple, provide for high yields, and allow for a highly purified product. Normally, the chemically reactive group will be created at the last stage, for example, with a carboxyl group, esterification to form an active ester will be the last step of the synthesis. Specific methods for the production of derivatized kringle 5 peptides of the present invention are described in examples below.

Each kringle 5 peptide selected to undergo the derivatization with a linker and a reactive agent will be modified according to the following criteria: if a carboxylic group, not critical for the retention of pharmacological activity is available on the original molecule and no other reactive functionality is present on the molecule, then the carboxylic acid will be chosen as attachment point for the linker-reactive group modification. If no carboxylic acids are available, then any other functionalities not critical for the retention of pharmacological activity will be selected as attachment point for the linker-reactive group modification. If several functionalities are available on kringle 5 peptide, a combination of protecting groups will be used in such a way that after addition of the linker/reactive group and deprotection of all the protected functional groups, retention of pharmacological activity is still obtained. If no reactive functionalities are available on the therapeutic agent, synthetic efforts will allow for a modification of the original parent drug in such a way that retention of biological activity and retention of receptor or target specificity is obtained.

The chemically reactive group is at a site, so that when the peptide is bonded to the blood component, the peptide retains a substantial proportion of the parent compound's inhibitor activity.

Even more specifically, each kringle 5 peptide selected to undergo the derivatization with a linker and a reactive group will be modified according to the following criteria: if a terminal carboxylic group is available on the kringle 5 peptide and is not critical for the retention of pharmacological activity, and no other sensitive functional group is present on the kringle 5 peptide, then the carboxylic acid will be chosen as attachment point for the linker-reactive group modification. If the terminal carboxylic group *is* involved in pharmacological activity, or if no carboxylic acids are available, then any other sensitive functional group not critical for the retention of pharmacological activity will be selected as the attachment point for the linker-reactive group modification. If several sensitive functional groups are available on a kringle 5 peptide,

a combination of protecting groups will be used in such a way that after addition of the linker/reactive entity and deprotection of all the protected sensitive functional groups, retention of pharmacological activity is still obtained. If no sensitive functional groups are available on the therapeutic peptide, [or if a simpler modification route is desired], synthetic efforts will allow for a modification of the original kringle 5 peptide in such a way that retention of biological activity and retention of receptor or target specificity is obtained. In this case the modification will occur at the opposite end of the peptide.

An NHS derivative may be synthesized from a carboxylic acid in absence of other sensitive functional groups in the kringle 5 peptide. Specifically, such a kringle 5 peptide is reacted with N-hydroxysuccinimide in anhydrous CH_2Cl_2 and EDC, and the product is purified by chromatography or recrystallized from the appropriate solvent system to give the NHS derivative.

Alternatively, an NHS derivative may be synthesized from a kringle 5 peptide that contains an amino and/or thiol group and a carboxylic acid. When a free amino or thiol group is present in the molecule, it is preferable to protect these sensitive functional groups prior to perform the addition of the NHS derivative. For instance, if the molecule contains a free amino group, a transformation of the amine into a Fmoc or preferably into a tBoc protected amine is necessary prior to perform the chemistry described above. The amine functionality will not be deprotected after preparation of the NHS derivative. Therefore this method applies only to a peptide whose amine group is not required to be freed to induce a pharmacological desired effect.

In addition, an NHS derivative may be synthesized from a kringle 5 peptide containing an amino or a thiol group and no carboxylic acid. When the selected molecule contains no carboxylic acid, an array of bifunctional linkers can be used to convert the molecule into a reactive NHS derivative. For instance, ethylene glycol-bis(succinimydylsuccinate) (EGS) and triethylamine dissolved in DMF

and added to the free amino containing molecule (with a ratio of 10:1 in favor of EGS) will produce the mono NHS derivative. To produce an NHS derivative from a thiol derivatized molecule, one can use N-[γ-maleimidobutyryloxy]succinimide ester (GMBS) and triethylamine in DMF. The maleimido group will react with the free thiol and the NHS derivative will be purified from the reaction mixture by chromatography on silica or by HPLC.

An NHS derivative may also be synthesized from a kringle 5 peptide containing multiple sensitive functional groups. Each case is have to be analyzed and solved in a different manner. However, thanks to the large array of protecting groups and bifunctional linkers that are commercially available, as described above, this invention is applicable to any peptide with preferably one chemical step only to derivatize the peptide or two steps by first protecting a sensitive group or three steps (protection, activation and deprotection). Under exceptional circumstances only, would one require to use multiple steps (beyond three steps) synthesis to transform a kringle 5 peptide into an active NHS or maleimide derivative.

A maleimide derivative may also be synthesized from a kringle 5 peptide containing a free amino group and a free carboxylic acid. To produce a maleimide derivative from a amino derivatized molecule, one can use N-[γ-maleimidobutyryloxy]succinimide ester (GMBS) and triethylamine in DMF. The succinimide ester group will react with the free amino and the maleimide derivative will be purified from the reaction mixture by crystallization or by chromatography on silica or by HPLC.

Finally, a maleimide derivative may be synthesized from a kringle 5 peptide containing multiple other sensitive functional groups and no free carboxylic acids. When the selected molecule contains no carboxylic acid, an array of bifunctional crosslinking reagents can be used to convert the molecule into a reactive NHS derivative. For instance maleimidopropionic acid (MPA) can be coupled to the free

amine to produce a maleimide derivative through reaction of the free amine with the carboxylic group of MPA using HBTU/HOBt/DIEA activation in DMF.

A large number of bifunctional compounds are available for linking to entities. Illustrative entities include: azidobenzoyl hydrazide, N-[4-(p-azidosalicylamino)butyl]-3'-[2'-pyridyldithio)propionamide), bis-sulfosuccinimidyl suberate, dimethyl adipimidate, disuccinimidyl tartrate, N-y-maleimidobutyryloxysuccinimide ester, N-hydroxy sulfosuccinimidyl-4-azidobenzoate, N-succinimidyl [4-azidophenyl]-1,3'-dithiopropionate, N-succinimidyl [4-iodoacetyl]aminobenzoate, glutaraldehyde, and succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate.

4. Uses of the Modified Kringle 5 Peptides

As described earlier, angiogenesis includes a variety of processes involving neovascularization of a tissue including "sprouting", vasculogenesis, or vessel enlargement. With the exception of traumatic wound healing, corpus leuteum formation and embryogenesis, it is believed that the majority of angiogenesis processes are associated with disease processes and therefore the use of the present therapeutic methods are selective for the disease and do not have deleterious side effects.

There are a variety of diseases in which angiogenesis is believed to be important, which may be treatable with the modified peptides of the invention. These diseases include, but not limited to, inflammatory disorders such as immune and non-immune inflammation, chronic articular rheumatism and psoriasis, disorders associated with inappropriate or inopportune invasion of vessels such as diabetic retinopathy, neovascular glaucoma, restenosis, capillary proliferation in atherosclerotic plaques and osteoporosis, and cancer associated disorders, such as solid tumors, solid tumor metastases, angiofibromas, retrolental fibroplasia, hemangiomas, Kaposi sarcoma and the like cancers which require neovascularization to support tumor growth.

The modified kringle 5 peptides of the invention find use in methods which inhibit angiogenesis in a diseased tissue ameliorates symptoms of the disease and, depending upon the disease, can contribute to cure of the disease. The modified peptides of the invention are more stable *in vivo* and, as such, smaller amounts of the modified peptide can be administered for effective treatment In one embodiment, the invention contemplates inhibition of angiogenesis, per se, in a tissue. The extent of angiogenesis in a tissue, and therefore the extent of inhibition achieved by the present methods, can be evaluated by a variety of method, for detecting α_5B_3-immunopositive immature and nascent vessel structures by immunohistochemistry.

As described herein, any of a variety of tissues, or organs comprised of organized tissues, can support angiogenesis in disease conditions including skin, muscle, gut, connective tissue, joints, bones and the like tissue in which blood vessels can invade upon angiogenic stimuli.

In one related embodiment, a tissue to be treated with the modified kringle 5 peptides of the invention is an inflamed tissue and the angiogenesis to be inhibited is inflamed tissue angiogenesis where there is neovascularization of inflamed tissue. In this class the method contemplates inhibition of angiogenesis in arthritic tissues, such as in a patient with chronic articular rheumatism, in immune or non-immune inflamed tissues, in psoriatic tissue and the like.

The patient treated in the present invention in its many embodiments is desirably a human patient, although it is to be understood that the principles of the invention indicate that the invention is effective with respect to all mammals, which are intended to be included in the term "patient." In this context, a mammal is understood to include any mammalian species in which treatment of diseases associated with angiogenesis is desirable, particularly agricultural and domestic mammalian species.

In another related embodiment, a tissue to be treated with the modified kringle 5 peptides of the invention is a retinal tissue of a patient with diabetic retinopathy, macular degeneration or neovascular glaucoma and the angiogenesis to be inhibited is retinal tissue angiogenesis where there is neovascularization of retinal tissue.

In an additional related embodiment, a tissue to be treated with the modified kringle 5 peptides of the invention is a tumor tissue of a patient with a solid tumor, a metastases, a skin cancer, a breast cancer, a hemangioma or angiofibroma and the like cancer, and the angiogenesis to be inhibited is tumor tissue angiogenesis where there is neovascularization of a tumor tissue. Typical solid tumor tissues treatable by the present methods include lung, pancreas, breast, colon, laryngeal, ovarian, and the like tissues.

Inhibition of tumor tissue angiogenesis is a particularly preferred embodiment because of the important role neovascularization plays in tumor growth. In the absence of neovascularization of tumor tissue, the tumor tissue does not obtain the required nutrients, slows in growth, ceases additional growth, regresses and ultimately becomes necrotic resulting in killing of the tumor.

The present invention thus provides for a method of inhibiting tumor neovascularization by inhibiting tumor angiogenesis according to the present methods using the modified kringle 5 peptides of the invention. Similarly, the invention provides a method of inhibiting tumor growth by practicing the angiogenesis-inhibiting methods. The methods are also particularly effective against the formation of metastases because (1) their formation requires vascularization of a primary tumor so that the metastatic cancer cells can exit the primary tumor and (2) their establishment in a secondary site requires neovascularization to support growth of the metastases.

In a related embodiment, the invention contemplates the practice of the method in conjunction with other therapies such as conventional chemotherapy directed against solid tumors and for control of

establishment of metastases. The administration of the modified kringle 5 peptides of the invention is typically conducted during or after chemotherapy, although it is preferably to inhibit angiogenesis after a regimen of chemotherapy at times where the tumor tissue will be responding to the toxic assault by inducing angiogenesis to recover by the provision of a blood supply and nutrients to the tumor tissue. In addition, it is preferred to administer the modified kringle 5 peptides after surgery where solid tumors have been removed as a prophylaxis against metastases. Insofar as the present methods apply to inhibition of tumor neovascularization, the methods can also apply to inhibition of tumor tissue growth, to inhibition of tumor metastases formation, and to regression of established tumors using the modified kringle 5 peptides of the invention.

Restenosis is a process of smooth muscle cell (SMC) migration and proliferation at the site of percutaneous transluminal coronary angioplasty which hampers the success of angioplasty. The migration and proliferation of SMC's during restenosis can be considered a process of angiogenesis which is inhibited by the modified kringle 5 peptides of the present invention. Therefore, the invention also contemplates inhibition of restenosis by inhibiting angiogenesis in a patient following angioplasty procedures. For inhibition of restenosis, the modified kringle 5 peptide is typically administered after the angioplasty procedure for from about 2 to about 28 days, and more typically for about the first 14 days following the procedure.

The present method for inhibiting angiogenesis in a tissue comprises contacting a tissue in which angiogenesis is occurring, or is at risk for occurring, with a composition comprising a therapeutically effective amount of a modified kringle 5 peptide. The dosage ranges for the administration of the modified kringle 5 peptide depend upon the form of the peptide, and its potency, as described further herein, and are amounts large enough to produce the desired effect in which angiogenesis and the disease symptoms mediated by angiogenesis are

ameliorated. The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

As angiogenesis inhibitors, such modified kringle 5 peptides are useful in the treatment of both primary and metastatic solid tumors and carcinomas of the breast; colon; rectum; lung; oropharynx; hypopharynx; esophagus; stomach; pancreas; liver; gallbladder; bile ducts; small intestine; urinary tract including kidney, bladder and urothelium; female genital tract including cervix, uterus, ovaries, choriocarcinoma and gestational trophoblastic disease; male genital tract including prostate, seminal vesicles, testes and germ cell tumors; endocrine glands including thyroid, adrenal, and pituitary; skin including hemangiomas, melanomas, sarcomas arising from bone or soft tissues and Kaposi's sarcoma; tumors of the brain, nerves, eyes, and meninges including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas and meningiomas; solid tumors arising from hematopoietic malignancies such as leukemias and including chloromas, plasmacytomas, plaques and tumors of mycosis fungoides and cutaneous T-cell lymphoma/leukemia; lymphomas including both Hodgkin's and non-Hodgkin's lymphomas; prophylaxis of autoimmune diseases including rheumatoid, immune and degenerative arthritis; ocular diseases including diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, retrolental fibroplasia, neovascular glaucoma, rubeosis, retinal neovascularization due to macular degeneration and hypoxia; abnormal neovascularization conditions of the eye; skin diseases including psoriasis; blood vessel diseases including hemagiomas and capillary proliferation within atherosclerotic plaques; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma;

wound granulation; diseases characterized by excessive or abnormal stimulation of endothelial cells including intestinal adhesions, Crohn's disease, atherosclerosis, scleroderma and hypertrophic scars (i.e. keloids) and diseases which have angiogenesis as a pathologic consequence including cat scratch disease (Rochele minalia quintosa) and ulcers (Helicobacter pylori). Another use is as a birth control agent which inhibits ovulation and establishment of the placenta.

The modified kringle 5 peptides of the present invention may also be useful for the prevention of metastases from the tumors described above either when used alone or in combination with radiotherapy and/or other chemotherapeutic treatments conventionally administered to patients for treating angiogenic diseases. For example, when used in the treatment of solid tumors, the modified kringle 5 peptides of the present invention may be administered with chemotherapeutic agents such as alpha inteferon, COMP (cyclophosphamide, vincristine, methotrexate and prednisone), etoposide, mBACOD (methortrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone), PRO-MACE/MOPP (prednisone, methotrexate (w/leucovin rescue), doxorubicin, cyclophosphamide, taxol, etoposide/mechlorethamine, vincristine, prednisone and procarbazine), vincristine, vinblastine, angioinhibins, TNP-470, pentosan polysulfate, platelet factor 4, angiostatin, LM-609, SU-101, CM-101, Techgalan, thalidomide, SP-PG and the like. Other chemotherapeutic agents include alkylating agents such as nitrogen mustards including mechloethamine, melphan, chlorambucil, cyclophosphamide and ifosfamide; nitrosoureas including carmustine, lomustine, semustine and streptozocin; alkyl sulfonates including busulfan; triazines including dacarbazine; ethyenimines including thiotepa and hexamethylmelamine; folic acid analogs including methotrexate; pyrimidine analogues including 5-fluorouracil, cytosine arabinoside; purine analogs including 6-mercaptopurine and 6-thioguanine; antitumor antibiotics including actinomycin D; the anthracyclines including doxorubicin, bleomycin,

mitomycin C and methramycin; hormones and hormone antagonists including tamoxifen and cortiosteroids and miscellaneous agents including cisplatin and brequinar. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy and kringle 5 administration with subsequent kringle 5 administration to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor.

5. Administration of the Modified Kringle 5 Peptides

The modified kringle 5 peptides will be administered in a physiologically acceptable medium, e.g. deionized water, phosphate buffered saline (PBS), saline, aqueous ethanol or other alcohol, plasma, proteinaceous solutions, mannitol, aqueous glucose, alcohol, vegetable oil, or the like. Other additives which may be included include buffers, where the media are generally buffered at a pH in the range of about 5 to 10, where the buffer will generally range in concentration from about 50 to 250 mM, salt, where the concentration of salt will generally range from about 5 to 500 mM, physiologically acceptable stabilizers, and the like. The compositions may be lyophilized for convenient storage and transport.

The subject modified kringle 5 peptides will for the most part be administered orally, parenterally, such as intravascularly (IV), intraarterially (IA), intramuscularly (IM), subcutaneously (SC), or the like. Administration may in appropriate situations be by transfusion. In some instances, where reaction of the functional group is relatively slow, administration may be oral, nasal, rectal, transdermal or aerosol, where the nature of the conjugate allows for transfer to the vascular system. Usually a single injection will be employed although more than one injection may be used, if desired. The modified kringle 5 peptides may be administered by any convenient means, including syringe, trocar, catheter, or the like. The particular manner of administration will vary depending upon the amount to be administered, whether a single bolus

or continuous administration, or the like. Preferably, the administration will be intravascularly, where the site of introduction is not critical to this invention, preferably at a site where there is rapid blood flow, e.g., intravenously, peripheral or central vein. Other routes may find use where the administration is coupled with slow release techniques or a protective matrix. The intent is that the kringle 5 peptide, analog or derivative be effectively distributed in the blood, so as to be able to react with the blood components. The concentration of the conjugate will vary widely, generally ranging from about 1 pg/ml to 50 mg/ml. The total administered intravascularly will generally be in the range of about 0.1 mg/ml to about 10 mg/ml, more usually about 1 mg/ml to about 5 mg/ml.

By bonding to long-lived components of the blood, such as immunoglobulin, serum albumin, red blood cells and platelets, a number of advantages ensue. The activity of the modified kringle 5 peptide compound is extended for days to weeks. Only one administration need be given during this period of time. Greater specificity can be achieved, since the active compound will be primarily bound to large molecules, where it is less likely to be taken up intracellularly to interfere with other physiological processes.

The formation of the covalent bond between the blood component may occur *in vivo* or *ex vivo*. For *ex vivo* covalent bond formation the modified kringle 5 peptide is added to blood, serum or saline solution containing human serum albumin or IgG to permit covalent bond formation between the modified kringle 5 peptide and the blood component. In a preferred format, the kringle 5 peptide is modified with maleimide and it is reacted with human serum albumin in saline solution. Once the modified kringle 5 peptide has reacted with the blood component, to form a kringle 5 peptide-protein conjugate, the conjugate may be administered to the patient.

Alternatively, the modified kringle 5 peptide may be administered to the patient directly so that the covalent bond forms between the modified kringle 5 peptide and the blood component in vivo.

6. **Monitoring the Pres nce of Modified Kringle 5 Peptide**

The blood of the mammalian host may be monitored for the presence of the modified kringle 5 peptide compounds one or more times. By taking a portion or sample of the blood of the host, one may determine whether the kringle 5 peptide has become bound to the long-lived blood components in sufficient amount to be therapeutically active and, thereafter, the level of kringle 5 peptide compound in the blood. If desired, one may also determine to which of the blood components the kringle 5 peptide derivative molecule is bound. This is particularly important when using non-specific kringle 5 peptides. For specific maleimide-kringle 5 peptides, it is much simpler to calculate the half life of serum albumin and IgG.

The modified kringle 5 peptides may be monitored using HPLC-MS or antibodies directed to kringle 5 peptides.

A. HPLC-MS

HPLC coupled with mass spectrometry (MS) can be utilized to assay for the presence of peptides and modified peptides as is well known to the skilled artisan. Typically two mobile phases are utilized: 0.1% TFA/water and 0.1% TFA/acetonitrile. Column temperatures can be varied as well as gradient conditions. Particular details are outlined in the Examples section below.

B. Antibodies

Another aspect of this invention relates to methods for determining the concentration of the kringle 5 peptides and/or analogs, or their derivatives and conjugates in biological samples (such as blood) using antibodies specific to the kringle 5 peptides or peptide analogs or their derivatives and conjugates, and to the use of such antibodies as a treatment for toxicity potentially associated with such kringle 5 peptides and/or their derivatives or conjugates. This is advantageous because the increased stability and life of the kringle 5 peptides *in vivo* in the patient might lead to novel problems during treatment, including

increased possibility for toxicity. The use of anti-therapeutic agent antibodies, either monoclonal or polyclonal, having specificity for a particular kringle 5 peptides, can assist in mediating any such problem. The antibody may be generated or derived from a host immunized with the particular modified kringle 5 peptide, or with an immunogenic fragment of the agent, or a synthesized immunogen corresponding to an antigenic determinant of the agent. Preferred antibodies will have high specificity and affinity for native, derivatized and conjugated forms of the modified kringle 5 peptide. Such antibodies can also be labeled with enzymes, fluorochromes, or radiolables.

Antibodies specific for modified kringle 5 peptides may be produced by using purified kringle 5 peptides for the induction of derivatized kringle 5 peptide-specific antibodies. By induction of antibodies, it is intended not only the stimulation of an immune response by injection into animals, but analogous steps in the production of synthetic antibodies or other specific binding molecules such as screening of recombinant immunoglobulin libraries. Both monoclonal and polyclonal antibodies can be produced by procedures well known in the art.

The antibodies may be used to monitore the presence of kringle 5 petides in the blood stream. Blood and/or surum samples may be analyzed by SDS-PAGE and western blotting. Such techniques permit the analysis of the blood or serum to determine the bonding of the modified kringle 5 peptides to blood components.

The anti-therapeutic agent antibodies may also be used to treat toxicity induced by administration of the modified kringle 5 peptide, and may be used *ex vivo* or *in vivo*. *Ex vivo* methods would include immuno-dialysis treatment for toxicity employing anti-therapeutic agent antibodies fixed to solid supports. *In vivo* methods include administration of anti-therapeutic agent antibodies in amounts effective to induce clearance of antibody-agent complexes.

The antibodies may be used to remove the modified kringle 5

peptides and conjugates thereof, from a patient's blood ex vivo by contacting the blood with the antibodies under sterile conditions. For example, the antibodies can be fixed or otherwise immobilized on a column matrix and the patient's blood can be removed from the patient and passed over the matrix. The modified kringle 5 peptides will bind to the antibodies and the blood containing a low concentration of the kringle 5 peptide, then may be returned to the patient's circulatory system. The amount of modified kringle 5 peptide removed can be controlled by adjusting the pressure and flow rate. Preferential removal of the modified kringle 5 peptides from the plasma component of a patient's blood can be effected, for example, by the use of a semipermeable membrane, or by otherwise first separating the plasma component from the cellular component by ways known in the art prior to passing the plasma component over a matrix containing the anti-therapeutic antibodies. Alternatively the preferential removal of kringle 5 peptide–conjugated blood cells, including red blood cells, can be effected by collecting and concentrating the blood cells in the patient's blood and contacting those cells with fixed anti-therapeutic antibodies to the exclusion of the serum component of the patient's blood.

The anti-therapeutic antibodies can be administered *in vivo*, parenterally, to a patient that has received the modified kringle 5 peptide or conjugates for treatment. The antibodies will bind the kringle 5 peptide compounds and conjugates. Once bound the kringle 5 peptide activity will be hindered if not completely blocked thereby reducing the biologically effective concentration of kringle 5 peptide compound in the patient's bloodstream and minimizing harmful side effects. In addition, the bound antibody–kringle 5 peptide complex will facilitate clearance of the kringle 5 peptide compounds and conjugates from the patient's blood stream.

The invention having been fully described is now exemplified by the following non-limiting examples.

EXAMPLES

General

Solid phase peptide synthesis of the Kringle-5 analogs on a 100 µmole scale was performed using manual solid-phase synthesis and a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1). When required, the selective deprotection of the Lys(Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). In some instances, the synthesis was then re-automated for the addition of one AEEA (aminoethoxyethoxyacetic acid) group, the addition of acetic acid or the addition of a 3-maleimidopropionic acid (MPA) (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The products were purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 µ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. Purity was determined 95% by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Exampl 1

Preparation of NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Lys-NH_2.3TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization

Example 2

Preparation of NAc-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2.3TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization.

Example 3

Pr paration of Nac-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-L u-Tyr-Asp-Tyr-Lys-NH_2.3TFA

Using automated peptide synthesis, the following protected

amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization.

Example 4

Preparation of NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2.4TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Ala-OH, Fmoc-Trp-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization.

Exampl 5

Preparation of NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Lys-NH_2.2TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Trp-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization

Example 6

Preparation of NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2.2TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Aloc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization.

The selective deprotection of the Lys(Aloc) group was performed

manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis was then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reversed phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 µ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ214 and 254 nm.

Example 7

Preparation of (MPA-AEEA)-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.2TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1).

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of Fmoc-AEEA. Deprotection of the resulting Fmoc-AEEA-peptid with piperidine 20% in DMF allow for the subsequent addition of the 3-MPA

(Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 µm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Example 8

Preparation of (MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.2TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1). Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a

Dynamax C_{18}, 60Å, 8 µm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Example 9

Preparation of NAc-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2.2TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Aloc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization

The selective deprotection of the Lys(Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis was then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5

mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ214 and 254 nm.

Example 10

Preparation of (MPA-AEEA)-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.2TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 μmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N*,*N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1).

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of Fmoc-AEEA. Deprotection of the resulting Fmoc-AEEA-peptide with piperidine 20% in DMF allow for the subsequent addition of the 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 μm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 μm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-

spray ionization.

Example 11

Preparation of (MPA)-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.2TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1 Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 µm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Exampl 12

Preparation of NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2.3TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Aloc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Ala-OH, Fmoc-Trp-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization.

The selective deprotection of the Lys(Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis was then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5

mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm.

Example 13

Preparation of (MPA-AEEA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.3TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 μmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1).

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Ala-OH, Fmoc-Trp-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of Fmoc-AEEA. Deprotection of the resulting Fmoc-AEEA-peptide with piperidine 20% in DMF allow for the subsequent addition of the 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 μm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 μm guard module, 21 mm

x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Example 14

Preparation of (MPA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.3TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1). Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Ala-OH, Fmoc-Trp-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH.

The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 µm guard module, 21 mm

x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Example 15

Preparation of NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Lys-(Nε-MPA)-NH_2.TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1).

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Aloc)-OH, Fmoc-Trp-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH. The selective deprotection of the Lys(Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis was then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5

mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ214 and 254 nm.

Example 16

Preparation of (MPA-AEEA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH_2.TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 μmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1).

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Trp-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of Fmoc-AEEA. Deprotection of the resulting Fmoc-AEEA-peptide with piperidine 20% in DMF allow for the subsequent addition of the 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 μm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 μm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Example 17

Preparation of (MPA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH_2.TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1). Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Trp-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 µm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Exampl 18

Pr paration of NAc-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2.2TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Aloc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization.

The selective deprotection of the Lys(Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis was then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm.

Exampl 19

Pr paration of (MPA-AEEA)-Arg-Lys-L u-Tyr-Asp-Tyr-NH_2.2TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide

on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1).

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of Fmoc-AEEA. Deprotection of the resulting Fmoc-AEEA-peptide with piperidine 20% in DMF allow for the subsequent addition of the 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 µm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization

Example 20

Preparation of (MPA)-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.2TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF)

solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1). Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 µm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Example 21

Preparation of NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Lys-(Nε-MPA)-NH_2.2TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Aloc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH. Deblocking of the Fmoc group the the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. Final cleavage from the resin was performed using cleavage mixture as described above. The product was isolated by precipitation and purified by preparative HPLC to afford

the desired product as a white solid upon lyophilization.

The selective deprotection of the Lys(Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis was then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 µ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm.

Example 22

Preparation of (MPA-AEEA)-Pro-Arg-Lys-Leu-Tyr-Asp-NH_2.2TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1).

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH (Step 1). The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of Fmoc-AEEA. Deprotection of the resulting Fmoc-AEEA-peptide with piperidine 20% in

DMF allow for the subsequent addition of the 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 µm guard module, 21 mm x 25 cm column an+d UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Example 23

Preparation of (MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-NH_2.2TFA

Solid phase peptide synthesis of the modified Kringle 5 peptide on a 100 µmole scale was performed on a Symphony Peptide Synthesizer using Fmoc protected Rink Amide MBHA resin, Fmoc protected amino acids, O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) in *N,N*-dimethylformamide (DMF) solution and activation with *N*-methyl morpholine (NMM), and piperidine deprotection of Fmoc groups (Step 1). Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH. The deprotection of the terminal Fmoc group is accomplished using 20% piperidine (Step 2) followed by the coupling of 3-MPA (Step 3). Resin cleavage and product isolation was performed using 86% TFA/5% TIS/5% H_2O/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax C_{18}, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax C_{18}, 60Å, 8 µm guard

module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm. The product had >95% purity as determined by RP-HPLC mass spectrometry using a Hewlett Packard LCMS-1100 series spectrometer equipped with a diode array detector and using electro-spray ionization.

Example 24

Preparation of NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε--AEEA-MPA)-NH_2.2TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Aloc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH (Step 1). Deblocking of the Fmoc group at the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling. The selective deprotection of the Lys(Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis was then re-automated for the addition of the AEEA (aminoethoxyethoxyacetic acid) group and of the 3-maleimidopropionic acid (MPA) (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm.

Example 25

Preparation of NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-$AEEA_n$-MPA)-NH_2.2TFA

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Aloc)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH (Step 1). Deblocking of the Fmoc group at the N-terminal of the resin-bound amino acid was performed with 20% piperidine in DMF for about 15-20 minutes. Coupling of the acetic acid was performed under conditions similar to amino acid coupling.

The selective deprotection of the Lys(Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin was then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis was then re-automated for the addition The synthesis was then re-automated for the addition of n AEEA (aminoethoxyethoxyacetic acid) groups and of the 3-maleimidopropionic acid (MPA) (Step 3). Resin cleavage and product isolation was performed using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et_2O (Step 4). The product was purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H_2O (A) and 0.045% TFA in CH_3CN (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm.

Example 26

P ptide Stability Assay

A peptide stability assay was performed. (MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-Lys-NH_2. 2TFA was synthesized as described above and was identified MPA-K5. The non-modified counterpart peptide Pro-Arg-Lys-Leu-Tyr-Asp-Lys-NH_2 was also synthesized as described above without the addition of 3-MPA and identified as K5.

K5 (MW = 1260.18, 918.12 freebase) was prepared as a 100 mM stock solution in water. MPA-K5 (MW = 1411.17, 1069.11 freebase) was prepared as a 100 mM stock solution in water. Human Serum Albumin (HSA) was obtained as a 25% solution (ca 250 mg/ml, 3.75 mM) as Albutein® available from Alpha Therapeutic. Human plasma was obtained from Golden West Biologicals.

a. Stability of K5 in human plasma

K5 was prepared as a lμM solution and dissolved in 25% human serum albumin. The mixture was then incubated at 37°C in the presence of human plasma to final concentration of 160 mM K5. Aliquots of 100 μl were withdrawn from the plasma at 0, 4 hours and 24 hours. The 100 μl aliquots were mixed with 100 μl of blocking solution (5 vol. 5%$ZnSO_4$/3 vol. Acetonitrile/2 vol. Methanol) in order to precipitate all proteins. The sample was centrifuged for 5 min at 10,000 g and the supernatant containing the peptide was recovered and filtered through a 0.22 μm filter. The presence of free intact K5 peptide was assayed by the HPLC/MS. The HPLC parameters for detection of K5 peptide in serum were as follows.

The HPLC method was as follows: A Vydac C18 250 X 4.6 mm, 5 μ particle size column was utilized . The column temperature was 30°C with a flow rate of 0.5 ml/min. Mobile Phase A was 0.1% TFA/water. Mobile Phase B was 0.1% TFA/acetonitrite. The injection volume was 10μl.

The gradient was as follows:

Time(Minutes)	%A	%B
0	95	5
20	70	30
25	10	90
30	10	90
35	95	5
45	95	5

The proteins were detected at 214, 254 and 334 nm. For mass spectral analysis, the ionization mode was API-electrospray (positive mode) at an M/Z range of 300 to 2000. The gain was 3.0, fragmentor 120v, threshold 20, stepsize 0.1. The gas temp was 350°C and the drying gas volume was 10.0 l/min. The Neb pressure was 24 psi and the Vcap was 3500V. The HPLC method was as follows: A Vydac C18 250 X 4.6 mm, 5 µ particle size column was utilized . The column temperature was 30°C with a flow rate of 0.5 ml/min. Mobile Phase A was 0.1% TFA/water. Mobile Phase B was 0.1% TFA/acetonitrite. The injection volume was 10µl.

The gradient was as follows:

Time(Minutes)	%A	%B
0	95	5
20	70	30
25	10	90
30	10	90
35	95	5
45	95	5

The proteins were detected at 214, 254 and 334 nm. For mass spectral analysis, the ionization mode was API-electrospray (positive mode) at an M/Z range of 300 to 2000. The gain was 3.0, fragmentor

120v, threshold 20, stepsize 0.1. The gas temp was 350°C and the drying gas volume was 10.0 l/min. The Neb pressure was 24 psi and the Vcap was 3500V.

Time	%K5 peptide in plasma
0 hrs.	100%
4 hrs	9%
24 hrs	0%

After only 4 hours incubation in plasma only 9% of the original K5 peptide remained. The results demonstrate that unmodified K5 peptide is unstable in serum likely as a result of protease activity.

b. Stability of MPA-K5-HSA Conjugate in Plasma

MPA-K5 (modified K5 peptide) was incubated with 25% HSA for 2 hours at room temperature. The MPA-K5-HSA conjugate was then incubated at 37° in the presence of human plasma at a final concentration of 160 µm. After the specific incubation period (0, 4 and 24 hours) an aliquot of 100 µl was withdrawn and filtered through a 0.22 µm filter. The presence of intact conjugate was assayed by HPLC-MS.

The column was an Aquapore RP-300, 250 x 4.6 mm, 7µ particle size. The column temperature was 50° C. The mobile phase A was 0.1% TFA/water. The mobile phase B was 0.1% TFA/acetonitrile. The injection volume was 1 µl. The gradient was as follows:

Time (minutes)	%A	%B	Flow(ml/min)
0	66	34	0.700
1	66	34	0.700
25	58.8	41.2	0.700
30	50	50	0.70
35	5	95	1.00
41	5	95	1.00
45	66	34	1.00
46	66	34	0.70

The peptide was detected at 214 mm for quantification. For mass spectral analysis of the peptide, the ionization mode was API-electrospray at 1280 to 1500 m/z range, gain 1.0, fragmentor 125V, threshold 100, stepsize 0.40. The gas temperature was 350°C the drying gas was 13.0 l/min. The pressure was 60psi and the Vcap was 6000V. The results are presented below.

Approximately 33% of circulating albumin in the bloodstream is mercaptalbumin (SH-albumin) which is not blocked by endogenous sulfhydryl compounds such as cysteine or glutathione and is therefore available for reaction with maleimido groups. The remaining 66% of the circulating albumin is capped or blocked by sulfhydryl compounds. The HPLC MS assay permits the identification of capped-HSA, SH-albumin and K5-MPA-albumin. The MPA covalently bonds to the free thiol on the albumin. The stability of the three forms of albumin in plasma is presented below.

Time	%capped HSA	% SH-Albumin	%K5-MPA-HSA
0 hrs.	61.3	16.6	22.1
4 hrs.	64.6	16.05	19.35
24 hrs.	63	16.8	20.2

The percentage of the three forms of human serum albumin remained relatively constant througout the 24 assay period. In particular, the percentage of K5-MPA-HSA remained relatively constant throughout the 24 hour plasma assay. These results are in dramatic contrast to the results obtained with unmodified K5 which decreased to 9% of the original amount of K5 in only 4 hours in plasma. The results demonstrate that, in contrast to K5 which is quite unstable in plasma, K5-MPA-HSA is quite stable from peptidase activity in plasma.

EXAMPLE 27

Endothelial Cell Migration Assay

The activity of modified anti-angiogenic peptides may be determined with an endothelial cell migration assay. The endothelial cell migration assay may be performed as described by Polverini, P. J. et al., Methods Enzymol, 198: 440-450 (1991), which is hereby incorporated herein by reference. Briefly, bovine capillary (adrenal) endothelial cells (BCE, which may be obtained from Judah Folkman, Harvard University Medical School) are starved overnight in DMEM containing 0.1% bovine serum albumin (BSA). Cells are then harvested with trypsin and resuspended in DMEM with 0.1% BSA at a concentration of 1.5×10^6 cells/mL. Cells are added to the bottom of a 48-well modified Boyden chamber (for example from Nucleopore Corporation, Cabin John, Md.). The chamber is assembled and inverted, and cells are allowed to attach for 2 hours at 37° C to polycarbonate chemotaxis membranes (5 μm pore size) that is soaked in 0.1% gelatin overnight and dried. The chamber is then reinverted and test substances are added to the wells of the upper chamber (to a total volume of 50 μl); the apparatus is then incubated for 4 hours at 37° C. Membranes are recovered, fixed and stained (DiffQuick, Fisher Scientific, Pittsburgh, Pa.) and the number of cells that have migrated to the upper chamber per 10 high power fields are counted. Background migration to DMEM+0.1% BSA may be subtracted and the data reported as the number of cells migrated per 10

high power fields (400 x) or when results from multiple experiments are combined, as the percent inhibition of migration compared to a positive control.

Example 28

Preparation of HSA-Kringle 5 Conjugates

Modified kringle 5 peptides are dissolved in distilled water to a final concentration of 100 mM. For the conjugation reaction, one volume of 100 mM modified kringle 5 peptide is added to 99 volumes of 25% HSA (Albutein®, 25% solution, Alpha Therapeutic inc.) to get 1 mM modified K5 : 3.75 mM HSA conjugates. The mixture is allowed to incubate at room temperature for 2 hours. The presence of the conjugate and the absence of unreacted modified K5 peptide are determined by HPLC coupled with mass spectrometry.

Example 29

Effect of Modified Kringle 5 peptides on Endothelial Cell Proliferation *In Vitro.*

The biological activity of free and HSA-conjugated Kringle 5 peptides may be determined *in vitro* using an endothelial cell proliferation assay. Bovine aortic endothelial cells are plated at a density of 2500 cells per well in a 96-well plate in Dulbecco's Modified Eagle medium (DMEM, Gibco) containing 10% heat inactivated calf serum. The cells were allowed to adhere for 24 hours, at 37°C in a 5% CO_2 incubator. The medium is then replaced with fresh DMEM (without serum) containing varying concentrations of inhibitor (free K5 peptide and HSA-Kringle 5 peptides). After 30 minutes at 37°C, bFGF (basic fibroblast growth factor) may then be added to a final concentration of 1ng/mL to stimulate growth. After 72 hours, the cell number may be measured using the colorimetric substrate WST-1 (Boehringer Mannheim) to determine the effect of modified K5 peptides on endothelial cell proliferation *in vitro*.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. A modified antiangiogenic peptide comprising a reactive group which reacts with amino groups, hydroxyl groups, or thiol groups on blood components to form stable covalent bonds wherein said reactive group is selected from the group consisting of succinimidyl and maleimido groups.

2. The as modified peptide of claim 1 wherein said peptide is a kringle 5 peptide.

3. A kringle 5 peptide according to claim 2 wherein said derivative is reactive with blood proteins.

4. A kringle 5 peptide according to claim 3, wherein the derivative is reactive with a thiol group on a blood protein.

5. A kringle 5 peptide according to claim 2 wherein the peptide is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9.

6. A kringle 5 peptide according to claim 2 wherein the peptide is selected from the group consisting of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15 and SEQ ID NO:16.

7. A composition comprising a derivative of kringle 5 peptide or analog thereof, said derivative comprising a reactive group which reacts with amino groups, hydroxyl groups or thiol groups on blood components to form stable covalent bonds wherein said reactive group is selected from the group consisting of succinimidyl and maleimido

groups for use in a method of treating angiogenesis in a human.

8. The composition of claim 7 wherein said derivative is reactive with blood proteins.

9. The composition of claim 7 wherein said derivative is reactive with a thiol group on a blood protein.

10. A derivative of a kringle 5 peptide, said derivative comprising a maleimido group which reacts with a thiol group on human serum albumin to form a covalent bond.

11. The derivative of claim 10 wherein said peptide is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9

12. The derivative of claim 10 wherein said peptide is selected from SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15 and SEQ ID NO:16.

13. A composition comprising a derivative of an anti-angiogenic peptide, said derivative comprising a maleimido group which reacts with a thiol group on human serum albumin to for a covalent bond for use in a method of treating angiogenesis in a human.

14. The composition of claim 13 wherein the peptide is a kringle 5 peptide.

15. The composition according to claim 14, wherein the peptide is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9.

16. A composition according to claim 14 wherein the peptide is selected from SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15 and SEQ ID NO:16.

17. Use of a composition for the manufacturer of a medicament extending the *in vivo* half-life of a kringle 5 peptide in a patient to provide an anti-angiogenic effect, the composition comprising a derivative of a kringle 5 peptide or analog thereof, said derivative comprising a reactive group which reacts with amino groups, hydroxo gropus, or thiol groups on blood components to form stable covalent bonds, wherein the reactive group is selected from the group consisting of succinimidyl and maleimido groups.

18. Use of a composition according to claim 14, wherein the derivative is reacted with blood proteins.

19. A modified kringle 5 peptide selected from the group consisting of NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Lys-NH_2; NAc-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2; Nac-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2; NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-NH_2; NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Lys-NH_2; NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2 and (MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.

20. A modified kringle 5 peptide selected from the group consisting of: NAc-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2; (MPA-AEEA)-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; MPA)-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2;

(MPA-AEEA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2; and
(MPA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Ala-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2.

21. 20. A modified kringle 5 peptide selected from the group consisting of NAc-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-Lys-(Nε-MPA)-NH_2; MPA-AEEA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH_2;
(MPA)-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH_2;
NAc-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-NH_2;
(MPA-AEEA)-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2;
(MPA)-Arg-Lys-Leu-Tyr-Asp-Tyr-NH_2;
NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Lys-(Nε-MPA)-NH_2;
(MPA-AEEA)-Pro-Arg-Lys-Leu-Tyr-Asp-NH_2;
(MPA)-Pro-Arg-Lys-Leu-Tyr-Asp-NH_2;
NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-AEEA-MPA)-NH_2; and
NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-$AEEA_n$-MPA)-NH_2.

SEQUENCE LISTING

<110> ConjuChem, Inc.
Beliveau, Richard
Bridon, Dominique
Rasamoelisolo, Michele
Thibaudeau, Karen
Huang, Xicai

<120> Long Lasting Anti-Angiogenic Peptides

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35 40 45

Ser Lys Glu Gln Gln Cys Val Ile Met Ala Glu Asn Arg Lys Ser Ser
50 55 60

Ile Ile Ile Arg Met Arg Asp Val Val Leu Phe Glu Lys Lys Val Tyr
65 70 75 80

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85 90 95

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100 105 110

His Arg Pro Arg Phe Ser Pro Ala Thr His Pro Ser Glu Gly Leu Glu
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130 135 140

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145 150 155 160

Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn Tyr Asp Gly Lys
165 170 175

Ile Ser Lys Thr Met Ser Gly Leu Glu Cys Gln Ala Trp Asp Ser Gln
180 185 190

Ser Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro Asn Lys Asn
195 200 205

Leu Lys Lys Asn Tyr Cys Arg Asn Pro Asp Arg Glu Leu Arg Pro Trp
210 215 220

Cys Phe Thr Thr Asp Pro Asn Lys Arg Trp Glu Leu Cys Asp Ile Pro
225 230 235 240

Arg Cys Thr Thr Pro Pro Pro Ser Ser Gly Pro Thr Tyr Gln Cys Leu
245 250 255

Lys Gly Thr Gly Glu Asn Tyr Arg Gly Asn Val Ala Val Thr Val Ser
260 265 270

Gly His Thr Cys Gln His Trp Ser Ala Gln Thr Pro His Thr His Asn
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Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu Asn Tyr Cys
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Arg Asn Pro Asp Gly Lys Arg Ala Pro Trp Cys His Thr Thr Asn Ser
305 310 315 320

Gln Val Arg Trp Glu Tyr Cys Lys Ile Pro Ser Cys Asp Ser Ser Pro
325 330 335

Val Ser Thr Glu Gln Leu Ala Pro Thr Ala Pro Pro Glu Leu Thr Pro
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Val Val Gln Asp Cys Tyr His Gly Asp Gly Gln Ser Tyr Arg Gly Thr
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Ser Ser Thr Thr Thr Thr Gly Lys Lys Cys Gln Ser Trp Ser Ser Met
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Thr Pro His Arg His Gln Lys Thr Pro Glu Asn Tyr Pro Asn Ala Gly
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Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala Asp Lys Gly Pro Trp
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Cys Phe Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Lys
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Lys Cys Ser Gly Thr Glu Ala Ser Val Val Ala Pro Pro Pro Val Val
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Leu Leu Pro Asp Val Glu Thr Pro Ser Glu Glu Asp Cys Met Phe Gly
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Pro Cys Gln Asp Trp Ala Ala Gln Glu Pro His Arg His Ser Ile Phe
485 490 495

Thr Pro Glu Thr Asn Pro Arg Ala Gly Leu Glu Lys Asn Tyr Cys Arg
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Asn Pro Asp Gly Asp Val Gly Gly Pro Trp Cys Tyr Thr Thr Asn Pro
515 520 525

Arg Lys Leu Tyr Asp Tyr Cys Asp Val Pro Gln Cys Ala Ala Pro Ser
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Phe Asp Cys Gly Lys Pro Gln Val Glu Pro Lys Lys Cys Pro Gly Arg
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Val Val Gly Gly Cys Val Ala His Pro His Ser Trp Pro Trp Gln Val
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Gly Thr Phe Gly Ala Gly Leu Leu Lys Glu Ala Gln Leu Pro Val Ile
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Ser Thr Glu Leu Cys Ala Gly His Leu Ala Gly Gly Thr Asp Ser Cys
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Ile Leu Gln Gly Val Thr Ser Trp Gly Leu Gly Cys Ala Arg Pro Asn
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BRIDON, Dominique, P.** [FR/CA]; 243 chemin Cote Ste-Catherine, Outremont, Quebec H2V 2B2 (CA). **RASAMOELISOLO, Michele** [MG/CA]; 1111 Mistral Avenue, Apt. 405, Montreal, Quebec H2P 2XS (CA). **THIBAUDEAU, Karen** [FR/CA]; 4700 Bonavista Street #407, Montreal, Quebec H3W 2L5 (CA). **HUANG, Xicai** [CA/CA]; 153 Denault, Kirkland, Quebec H9J 3X2 (CA). **BELIVEAU, Richard** [CA/CA]; 266 Wilson Avenue, Verdun, Quebec H3E 1L8 (CA).

(74) Agent: **BERESKIN & PARR**; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
19 April 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/70665 A3

(54) Title: LONG LASTING ANTI-ANGIOGENIC PEPTIDES

(57) Abstract: Modified anti-angiogenic peptides are disclosed. The modified peptides are capable of forming a peptidase stabilized anti-angiogenic peptide. The modified anti-angiogenic peptides, particularly modified kringle 5 peptides are capable of forming a conjugate with a blood protein. Conjugates are prepared from anti-angiogenic peptides, particularly kringle 5 peptides, by combining the peptide with a reactive functional group with a blood protein. The conjugates may be formed *in vivo* or *ex vivo*. The conjugates are administered to patients to provide an anti-angiogenic effect.

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
REDC-2200PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IB 00/00763	17/05/2000	17/05/1999
Applicant		
CONJUCHEM, INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/00763

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 0602290	A	22-06-1994	NONE		
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			AU	1519699 A	31-05-1999
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			US	6057122 A	02-05-2000
WO 0004052	A	27-01-2000	AU	5049599 A	07-02-2000

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted]B 00/00763

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/68 A61K38/48 A61P7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 602 290 A (POULETTY PHILIPPE ;POULETTY CHRISTINE (US)) 22 June 1994 (1994-06-22) column 5, line 32 - line 53 column 6, line 44 - line 55; claims ---	1
P,X	WO 99 48536 A (CONJUCHEM INC ;HOLMES DARREN L (CA); BRIDON DOMINIQUE P (CA); EZRI) 30 September 1999 (1999-09-30) page 6, line 13 - line 17 page 8, line 11 - line 15 page 24, line 15 -page 25, line 2; claims; example 25 ---	1,13
P,A	WO 99 24074 A (CONJUCHEM INC ;HOLMES DARREN L (CA); BRIDON DOMINIQUE P (CA); EZRI) 20 May 1999 (1999-05-20) claims; examples ---	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 December 2000	21/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted] 00/00763

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 99 24075 A (CONJUCHEM INC ;HOLMES DARREN L (CA); BRIDON DOMINIQUE P (CA); HUAN) 20 May 1999 (1999-05-20) page 7, paragraph 2 page 10, paragraph 1; claims; examples	1
X	WO 97 23500 A (CHILDRENS MEDICAL CENTER) 3 July 1997 (1997-07-03) claims; examples	5,6, 19-21
X	WO 97 41824 A (ABBOTT LAB) 13 November 1997 (1997-11-13) claims; examples	5,6, 19-21
P,X	WO 00 04052 A (BRIGHT JEREMY RICHARD ;ADPROTECH PLC (GB); STEWARD MICHAEL (GB); C) 27 January 2000 (2000-01-27) claims; examples	5,6, 19-21

1